INSIGHTS INTO THE NATURE AND IMMUNE ESCAPE OF A TRANSMISSIBLE CANCER IN TASMANIAN DEVILS

César Darío Tovar López,
BSc(Hons) GradDipEnvSt(Hons)

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

University of Tasmania
July 2012

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

César D. Tovar López

Authority of Access

César D. Tovar López

Statement of Co-Authorship

(for school files and submission with Thesis Submission Form)

Paper 1.

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Murchison, E. P., Tovar, C., Hsu, A., Bender, H. S., Kheradpour, P., Rebbeck, C. A., Obendorf, D., Conlan, C., Bahlo, M., Blizzard, C. A., Pyecroft, S., Kreiss, A., Kellis, M., Stark, A., Harkins, T. T., Marshall Graves, J. A., Woods, G. M., Hannon, G. J., Papenfuss, A. T.

Title

The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally transmissible cancer.

Journal

Science 2010: 327(5961): 84-87.

The following people contributed to the publication of the work undertaken as part of this thesis:

Candidate - Tovar (10%) – produced one of the three figures in the main text

First and corresponding author - Murchison (50%)
The other authors contributed to the remaining 40%
Murchison, Tovar, Woods, Hannon and Papenfuss contributed to formulation, development and manuscript preparation
Murchison, Tovar and Papenfuss contributed to the majority of data collection
Remaining authors contributed to data collection, design and manuscript refinement

We the undersigned agree with the above stated "proportion of work undertaken" for the above published peer-reviewed manuscripts contributing to this thesis:

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Corresponding Author - Murchison _____

Paper 2.
Authors
Kreiss A, Tovar C, Obendorf DL, Dun K, Woods GM
Title
A murine xenograft model for a transmissible cancer in Tasmanian devils.
Journal
Veterinary Pathology 2011 Mar (2):475-81

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Candidate - Tovar (25%) – Produced one of four figures in the text
Obendorf (5%)
Dun (10%),
Woods (10%)

Kreiss and Woods contributed to formulation design, development and manuscript preparation
Kreiss, Tovar and Dun contributed to data acquisition and laboratory work
Kreiss, Tovar Obendorf contributed to histological assessment
All authors contributed to manuscript refinement

We the undersigned agree with the above stated "proportion of work undertaken" for the above published peer-reviewed manuscripts contributing to this thesis:

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Paper 3.
Authors
Tovar C, Obendorf D, Murchison EP, Papenfuss AT, Kreiss A, Woods GM.
Title
Tumor-specific diagnostic marker for transmissible facial tumors of Tasmanian devils: immunohistochemistry studies.
Journal
Veterinary Pathology 2011: 48(6):1195-203

The following people contributed to the publication of the work undertaken as part of this thesis:

First author and *Candidate Tovar (60%) Produced all the tables and figures for the manuscript*
Obendorf (15%)
Murchison (5%)
Papenfuss (5%)
Kreiss (5%)
Corresponding Author Woods (10%)

We the undersigned agree with the above stated "proportion of work undertaken" for the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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Corresponding Author - Woods _____

Tovar and Woods contributed to formulation design, development and manuscript preparation
Tovar was responsible for the data collection and laboratory work
Tovar, Obendorf and Kreiss contributed to analysis of results
Murchison and Papenfuss contributed to the genetics and bioinformatics
All authors contributed to manuscript refinement.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____ _____

A/Prof Greg Woods
Supervisor
Menzies Research Institute Tasmania
University of Tasmania

Prof Alison Venn
Acting Director
Menzies Research Institute Tasmania
University of Tasmania

Date: 15/02/2012

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Abstract

Devil facial tumour disease (DFTD) is a transmissible cancer that threatens the survival of the Tasmanian devil (*Sarcophilus harrisii*), the world's largest living carnivorous marsupial. Despite devils having a competent immune system, there is no evidence of natural immunity to the tumours. Affected animals die within months of tumour appearance. The species could face extinction within 25 to 35 years. Cytogenetic and molecular studies have confirmed that the infectious agent is the cancer cell itself. However, our knowledge about the nature and biology of this tumour is limited.

Tumour transcriptome analyses of DFTD tumours and devil tissues revealed that DFTD expresses a set of genes related to the myelination pathway in the peripheral nervous system. This thesis examined the protein expression of peripheral nerve and other neuronal markers in DFTD to further elucidate the nature of the tumour. It also evaluated the utility of neuronal and peripheral nerve proteins as diagnostic markers for the disease. Immunohistochemistry and flow cytometry confirmed that DFTD tumour cells express a number of proteins found throughout the neuronal system. Notably, DFTD tumour cells expressed structural proteins found in myelinating Schwann cells in the peripheral nervous system. These included peripheral myelin protein 22 (PMP22), myelin protein zero (MPZ), myelin basic protein (MBP), and the recently described periaxin (PRX). Nerve growth factor receptor (NGFR), which is involved in the differentiation of Schwann cells, was also detected in DFTD tumour cells. These results coupled with the available genetic data confirm that DFTD cells are of Schwann cell origin.

Furthermore, this study showed that periaxin, a specific marker of Schwann cells, is consistently expressed in DFTD primary tumours, DFTD metastases, DFTD cell lines and murine xenografted DFTD tumours. Therefore, this thesis identified periaxin as a sensitive and specific marker for DFTD. This will greatly facilitate the diagnosis of the disease.

How DFTD can be transplanted across major histocompatibility (MHC) barriers is an aspect of DFTD that is not understood. Initially this was attributed to the reduced

diversity at the major histocompatibility complex (MHC) loci. However, recent studies showed that the limited MHC diversity in devils is sufficient to produce measurable mixed lymphocyte reactions and the rejection of skin allografts. Thus, this thesis investigated the expression of MHC to determine if this may explain the lack of immune response to DFTD cells.

Analysis of the expression of MHC proteins in devils has been hindered by the lack of cross-reacting reagents. During the course of this study collaborators from the University of Cambridge (UK) developed the first anti-devil MHC class I (MHC-I) antibody. This antibody was used to examine the expression of MHC-I in devil tissues and DFTD. MHC-I protein expression in primary tumours and metastases was lower than normal tissues. Its expression was also variable within a tumour and among different tumours. These results suggest that alteration of MHC-I expression could contribute to the immune escape of DFTD.

Immunisation trials with non-viable DFTD cell lines revealed that some Tasmanian devils can produce antibodies against DFTD cells. This thesis utilised an immunoproteomic approach to identify immunogenic proteins in DFTD. Several candidates were identified as DFTD tumour associated antigens. These include heat shock proteins, tubulin, histone H2B, stathmin protein, and proliferating cell nuclear antigen (PCNA). These proteins are excellent targets for the development of early diagnostic tools as well as therapeutic approaches.

In summary, this thesis provided strong supporting evidence for a Schwann cell origin of DFTD and generated a specific diagnostic marker for the disease. Additionally, this thesis opened new opportunities for the understanding of the mechanisms that allow immune evasion and the interaction between the tumour cells and the devil's immune system. These results will have direct implications for the future development of a vaccine approach.

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Special thanks to Alex Kreiss for his friendship and for re-introducing me to the scientific realm and this fascinating research. We have learnt together, shared frustrations and gratifying experiences through our work. Thank you so much.

This research would have not been possible without the work and collaboration of friends, colleagues and staff from the Save the Tasmanian devil Program, the Tasmanian Department of Primary Industry, Parks, Water and Environment, Mount Pleasant Laboratory and the University of Tasmania. This research was possible thanks to the sampling material collected and provided by the team. Special thanks to Dr Colette Harmesen, Dr Sarah Peck, Dr Rodrigo Hamede and devil keepers. I appreciate all your support and collaboration.

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Working at Menzies Institute Tasmania gave me the opportunity to meet wonderful people. Thanks to my friends in the Immunology Group, Heather, Roslyn, Nick, Gabby, Jess, Terry, Lauren and Takumi. Thank you to Niall, Kathy, Ruth, Stan, Olivier, Jerome and Naseem for all your friendship, support and assistance.

Thanks to my parents, family and old friends. Your support, encouragement, and energy have kept me motivated through all my journeys far from home. I am immensely grateful.

Abbreviations

APC	Antigen presenting cell
APM	Antigen processing machinery
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CNS	Central nervous system
CTVT	Canine venereal transmissible tumour
DFTD	Devil facial tumour disease
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DPIPWE	Department of Primary Industry, Parks, Water and Environment
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FCS	Foetal calf serum
H&E	Hematoxylin and eosin
HRP	Horseradish peroxidase
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
MAGE-1	Melanoma antigen
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MPNST	Malignant peripheral nerve tumour
NCC	Neural crest cells
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NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
NGFR	Nerve growth factor receptor
NK	Natural killer cell
NSE	Neuron specific enolase
PBS	Phosphate buffered saline
PMP22	Peripheral myelin protein 22
PMPZ	Peripheral myelin protein zero
PNS	Peripheral nervous system
PNST	Peripheral nerve sheath tumour
PRX	Periaxin
S100	S-100 protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAAs	Tumour-associated antigens
TAP-1	Peptide transporter protein-1
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1 Introduction

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1.1 Introduction

Devil facial tumour disease (DFTD) is a recently emerged transmissible cancer that is threatening the survival of the Tasmanian devil (*Sarcophilus harrisii*), the largest extant carnivorous marsupial (Hawkins *et al.*, 2006, McCallum and Jones, 2006). Primary tumours located around the mouth and face that grow to in excess of 10 cm in diameter and often ulcerate characterize this cancer. Karyotypic analysis of DFTD tumours has revealed a substantial and consistent pattern of chromosomal abnormalities from different animals. This similarity led to the proposal that DFTD is transmitted as an allograft (Pearse and Swift, 2006). Genetic analysis of DFTD tumours at the DNA, microsatellite, and major histocompatibility complex loci, provided further support for the clonal transmission and therefore the contagious nature of this tumour (Murchison *et al.,* 2010, Siddle *et al.,* 2007b). DFTD transmission occurs by facial biting with the transfer of viable cancer cells dislodged from ulcerated tumours in close proximity to the canine teeth (Obendorf and McGlashan, 2008).

There is no evidence of natural immunity to the establishment and growth of the tumours, and affected animals die within months of tumour appearance (Hawkins *et al.,* 2006, Woods *et al.,* 2007). At the current rate of spread of disease, the species could face extinction within 25 to 35 years (McCallum *et al.,* 2007). Conservation management trials have already begun (http://www.tassiedevil.com.au/tasdevil.nsf/). These include the removal of DFTD-affected devils from relatively isolated local populations in Tasmania and the establishment of insurance populations of healthy devils in mainland Australia, in free-range enclosures and in islands off the Tasmanian mainland

DFTD was initially described as a malignant neuroendocrine neoplasm (Loh *et al.,* 2006b). Recent studies on the Tasmanian devil transcriptome supported the neuroectodermal origin and revealed that DFTD expresses a set of genes related to the myelination pathway in the peripheral nervous system (PNS) (Murchison *et al.,* 2010). Analyses of protein expression are required to confirm the nature of the tumour and to identify potential diagnostic biomarkers for the disease.

DFTD is a unique cancer as it is transmitted between unrelated individuals without evoking an immune response (Woods *et al.,* 2007). The lack of immune allorecognition was initially attributed to the reduced genetic diversity that characterises the devil populations and the lack of genetic diversity at the major histocompatibility complex (MHC) loci (Siddle *et al.,* 2007a). However, recent studies showed that the limited MHC diversity is sufficient to produce measurable mixed lymphocyte reactions and the rejection of skin allografts (Kreiss *et al.*, 2011a). Therefore, the mechanisms that explain the lack of immune response to the transfer of DFTD cells require further investigation.

Canine transmissible venereal tumour (CTVT) is another naturally occurring contagious cancer that may provide clues about the mechanisms that allow transplantation of cells across MHC barriers. CTVT is sexually transmitted and affects dogs worldwide (Rebbeck *et al.,* 2009). However, CTVT is not a fatal cancer. Initial transplantation of CTVT tumour cells occurs through the downregulation of MHC molecules and immune suppression induced by the tumour. This is followed by a complex interaction between the cancer cells and the dog's immune system that ends with the regression of the tumours (reviewed in Murchison, 2008). Hence, CTVT provides a useful model for the study of the mechanisms of immune evasion in DFTD.

Understanding the circumstances that allow transmission of DFTD is also essential for the development of therapeutic strategies. Immunisation trials with non-viable DFTD cell lines revealed that Tasmanian devils are able to produce a humoral (antibody) response against the cancer cells. The identification of the specific antigenic and immunogenic proteins in DFTD is of paramount importance. It will open new opportunities for the understanding of the interaction between the tumour cells and the devil's immune system and have direct implications for the future development of a vaccine approach.

1.2 Transmissible cancers

Specific infectious agents including viruses, bacteria and parasites can cause cancer, however, the disease per se is not generally considered transmissible (Zur Hausen, 2009). In humans, there is no evidence for direct person-to-person transmission of tumour cells during normal social interaction (Dingli and Nowak, 2006). The transmission of cancer between individuals occurs only under very particular biological settings. Pregnancy and foetal to foetal transmission are the only known physiological routes of tumour cell transmission in humans (Dessolle *et al.,* 2007). Organ or hematopoietic cell transplantation are other possible route of cancer transmission between people (Birkeland and Storm, 2002, Brunstein *et al.*, 2002, Buell *et al.*, 2004, Gandhi and Strong, 2007, Strauss and Thomas, 2010, Wang *et al.*, 2011). However, these cases are extremely rare and less than 0.06% of recipients of transplants develop cancer from the transferred organ or cells (Feng *et al.,* 2003, Kauffman *et al.,* 2002). Finally, there is a case report of a surgeon developing a malignant histiocytoma after an accidental injury during the surgical removal of a tumour from his unrelated patient (Gartner *et al.*, 1996).

The fact that transmission of cancer between people is very rare supports the notion that the immune system prevents such cancers to take. In humans and other jawed vertebrates, specialised proteins encoded by genes called the major histocompatibility complex (MHC) are responsible for graft rejection. The immune system uses MHC antigens to differentiate “self” from “non-self” because these proteins vary between individuals. The immune system reacts against those cells displaying non-self MHC antigens (Bradley, 1991). Therefore, MHC genes play a critical role in protecting vertebrate species from infectious diseases and contagious cancers (Belov, 2011).

There are only two cases of naturally occurring contagious cancers: the canine transmissible venereal tumour (CTVT) and devil facial tumour disease (DFTD). CTVT is a sexually transmissible tumour in dogs that originated thousands of years ago (Murgia *et al.,* 2006, Rebbeck *et al.,* 2009). DFTD is an aggressive facial tumour that emerged no more than 20 years ago and is threatening the survival of the Tasmanian devil in the wild (Hawkins *et al.,* 2006). CTVT and DFTD tumour cells behave like infectious parasites passing from one host to another across MHC barriers. Although

these two cancers have similar origin, the host immune response and adaptation are unique. CTVT and DFTD are therefore excellent models to study tumour immunology and the evolution of infectious cancers.

1.2.1 Canine venereal transmissible tumour

Canine transmissible venereal tumour (CTVT) is a sexually transmitted neoplasm of domestic dogs (*Canis familiaris*). CTVT causes lesions around the external genitalia and affects both sexes of any breed of dog (Figure 1.1) (Brown *et al.*, 1980, Karlson and Mann, 1952). Tumours are transferred and maintained in the dog population by coitus and other social behaviours such as smelling and licking of the external genitalia (Das and Das, 2000, Higgins, 1966, Ndiritu *et al.*, 1977). The disease is globally widespread, particularly in subtropical countries, with cases reported in North, Central and South America, Europe, Middle and Far East, Asia and parts of Africa (Kabuusu *et al.,* 2010).

Cytologically, CTVT tumours appear as compact masses of neoplastic cells arranged in a diffuse pattern and supported by a thin fibro vascular tissue. The tumour cells are round or polyhedral with a prominent nucleus and a slightly granular and vacuolated cytoplasm (Karlson and Mann, 1952, Mukaratirwa and Gruys, 2003). The nature and cell of origin of is not yet clear but histological studies suggested that CTVT evolved from a cell of the macrophage lineage (Marchal *et al.,* 1997, Mozos *et al.,* 1996).

Figure 1.1 Ulcerated CTVT tumour in a female dog. Image from: http://www.carefordogs.org

Infectious nature of the CTVT

For the first time in 1876, Nowinsky demonstrated the transmissible nature of CTVT by transplanting the tumour from one dog to another (Nowinsky, 1876). Similarly, Karlson and Mann (1952) passaged the tumour through 40 generations of dogs over a period of

7 years. Further studies confirmed that transmission of CTVT occurs only by transplantation of living tumour cells and not by killed cells or cellular filtrates (Koike *et al.*, 1979, Rust, 1949, Stubbs and Furth, 1934). CTVT was also experimentally transmitted to other related canids, such as the coyote (*Canis latrans*) (Cockrill and Beasley, 1979) and the grey wolf (*Canis lupus*) (Karlson and Mann, 1952, Wade, 1908).

The success of transplantation of CTVT tumours between unrelated dogs led to the proposal that the disease is a contagious cancer transmitted as an allograft. Cytogenetic studies on spontaneous and experimentally transplanted CTVT supported this theory. Studies of tumours from different geographical locations showed that the tumour cells were always aneuploidy and had a comparable number of chromosomes. They also had similar frequency of metacentric chromosomes and similar incidence of marker chromosomes (reviewed in Mukaratirwa and Gruys, 2003). The remarkable similarity between CTVT karyotypes, which clearly differ from that of the normal canine cell, suggests not only a common origin for CTVT but also highlights the stable nature of the clone (Murchison, 2008).

Recent genetic studies provided further evidence for the clonal transmission of CTVT. CTVT tumours have a long interspersed nuclear element (LINE-1) inserted near the *MYC* gene that is not found in any other canine tissue. This element is now a diagnostic marker for CTVT (Katzir *et al.,* 1987, Liao *et al.,* 2003b).

Murgia *et al.* (2006) and Rebbeck *et al.* (2009, 2011) used microsatellite diversity to analyse the origin and evolution of CTVT. These studies provided the conclusive evidence for a monophyletic origin of CTVT. They confirmed that tumours are closely related genetically but genetically different from their host. Moreover, they predicted that CTVT emerged in inbred wolves more than 6000 years ago, probably when dogs were first domesticated. In contrast, the common ancestor of existing tumours probably appeared only around 47 and 470 years ago (Rebbeck *et al.,* 2009) or between 250 and 2500 years ago (Murgia *et al.*, 2006).

Tumour immunology

Typically, CTVT is a benign disease and the progression of CTVT tumours follows a predictable growth pattern. In natural and experimental cases, the tumour grows rapidly after transplantation, followed by a stable phase, and finally a spontaneous regression phase, generally within six months after the first appearance (Higgins, 1966, Karlson and Mann, 1952). Metastases are rare and generally occur in puppies and immunosuppressed dogs (Cohen, 1985). Dogs in which tumours regressed spontaneously are resistant to further transmission of CTVT (Karlson and Mann, 1952).

The foreseeable growth of tumours and the frequent spontaneous regression of CTVT reveal two important aspects of the disease. First, CTVT has evolved to be an effective transmissible parasite. Second, it indicates that the dog's immune response plays a major role in defining the course of the disease (Mukaratirwa and Gruys, 2003, Murgia *et al.*, 2006). However, the mechanisms of this process are not completely clear.

CTVT triggers both humoral and cell-mediated immune responses in the host (reviewed in Murchison, 2008). However, during the progression phase the tumour cells evade immune recognition by different mechanisms. CTVT produces high concentrations of TGF-β which decreases MHC expression and suppresses natural killer cell activity (Cohen *et al.,* 1984, Hsiao *et al.,* 2002, Hsiao *et al.,* 2004, Murgia *et al.,* 2006); CTVT also secretes substances that destroy B-cells (Liao *et al.,* 2003a), and prevents the differentiation and activity of dendritic cells (Liu *et al.,* 2008).

During the progression phase, specific circulating antibodies to CTVT tumour antigens can be detected in affected animals (Cohen, 1972, McKenna and Prier, 1966). However, the levels of antibodies detected in the serum do not correlate well with tumour growth. This indicates that the antibodies are not protective against established CTVT. Instead, the humoral response appears to have a more important role in slowing the tumour growth, protecting against metastasis, and reducing the susceptibility to future CTVT infections (Cohen, 1980, Fenton and Yang, 1988).

After the progression phase, the tumour growth slows and becomes vulnerable to the host immune response. A proposed model suggests that during the regression phase, the number of tumour-infiltrating lymphocytes increases. These cells secrete IFN-γ and

interleukin-6 (IL-6) which block the inhibitory effects of tumour-derived TGF-β and induce the expression of MHC in tumour cells. The expression of MHC by CTVT triggers the immune system and the regression of the tumour (Hsiao *et al.,* 2004).

The fine-tuned interactions between CTVT and the host reveal a process of coevolution where the parasite and the host's immune system have adapted to each other strategies. The notion of a cancer somatic cell evolving to become infectious and survive thousands of years is intriguing. Certainly, CTVT will continue to provide us with a better understanding of the role of the immune system in the evolution of such cancers.

1.2.2 Devil facial tumour disease

Tasmanian devils (*Sarcophilus harrisii*) are the world's largest extant marsupial carnivores and are endemic to Tasmania, Australia's island state. Tasmanian devil populations were isolated from those on mainland approximately 12000 years ago at the end of the last glaciation (Turney *et al.*, 2008). The continental populations then became extinct approximately 400 years ago, probably due to competition with the dingo (*Canis lupus*) and humans (Johnson, 2003). Tasmanian devils have moderately low genetic diversity, possibly due to the island effect and repeated periods of low population density over the past 150 years (Jones *et al.,* 2004, Miller *et al.,* 2011, Siddle *et al.,* 2007a). Although devils are specialist carrion feeders, they have played the role of principle native carnivore since the extinction of the thylacine (*Thylacinus cynocephalus*).

Until the emergence of Devil Facial Tumour Disease (DFTD) in 1996, Tasmanian devils were common and found throughout the island (McCallum *et al.,* 2007). This unusual transmissible cancer has spread through the island producing substantial declines in devil populations. In Tasmania's north-east, where the disease first appeared, spotlighting sightings declined by 80% (Hawkins *et al.,* 2006). Other local populations have declined by at least 60% (Lachish *et al.,* 2007). At the current rate of spread of the disease it is estimated that species could face extinction within 25-35 years (McCallum *et al.,* 2007). The Australian and State Government listed the Tasmanian devil as Endangered under environmental law. The species was also listed as Endangered on the

Red List of the International Union for the Conservation of Nature and Natural Resources (IUCN).

Clonal transmission of DFTD

Similar to CTVT, karyotypic analysis of DFTD tumours from different locations revealed a substantial and consistent pattern of chromosomal abnormalities among the DFTD tumours (Figure 1.2). Moreover, a pericentric inversion of chromosome 5 was found in the constitutional karyotype of a devil, but such inversion was not observed in either chromosome 5 of the tumour from this animal. These findings led to the proposal that DFTD is transmitted as an allograft (Pearse and Swift, 2006). Genetic analysis of DFTD tumours at the DNA, microsatellite and major histocompatibility complex loci provided further support for clonal transmission and therefore the contagious nature of this tumour (Miller *et al.,* 2011, Murchison *et al.,* 2010, Siddle *et al.,* 2007b). Facial biting with the transfer of viable tumour cells dislodged from ulcerated tumours in close proximity to the canine teeth is considered the most likely means of DFTD transmission (Obendorf and McGlashan, 2008).

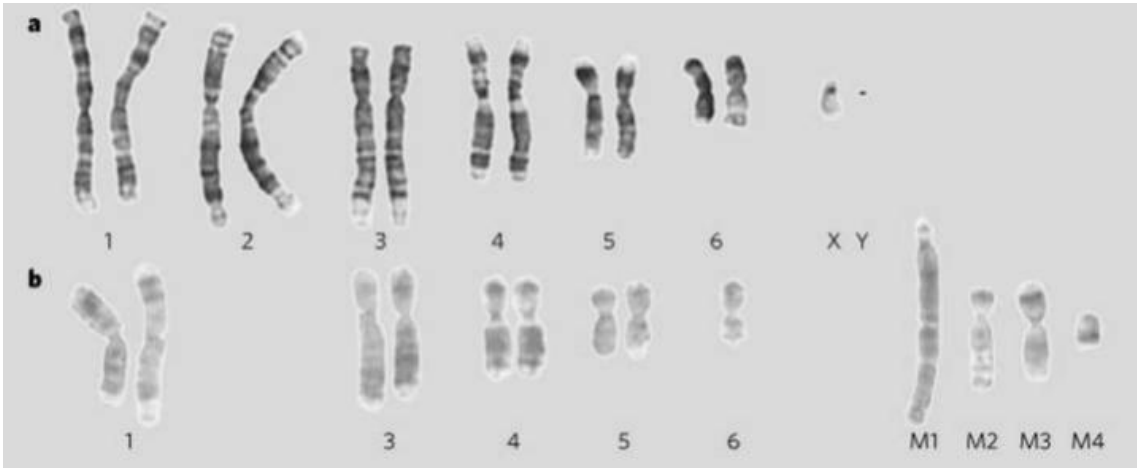

Figure 1.2 Karyotype of the Tasmanian devil and DFTD cells. **a**, Normal karyotype of a male Tasmanian devil (14 chromosomes, including XY). **b**, Karyotype of cancer cells (13 chromosomes; sex chromosomes, chromosome 2 pair and one chromosome 6 are lost; four additional marker chromosomes are present (M1–M4). (Image taken from Pearse and Swift, 2006)

Immunology of DFTD

Contrasting to CTVT, there is no evidence of host resistance to the establishment and growth of DFTD tumours and affected animals die within months of tumour appearance (Hawkins *et al.*, 2006, Kreiss *et al.*, 2008, Woods *et al.*, 2007).

Knowledge of the devil's immune system was limited before the emergence of DFTD and the lack of response against DFTD was initially attributed to a severely compromised immune system. However, *in vivo* and *in vitro* studies in our laboratory demonstrated that devils have a competent immune system:

- Devils have the full complement of secondary lymphoid organs with a distribution of immune cells similar to eutherian animals. These include neutrophils, lymphocytes, monocytes, basophils and eosinophils (Kreiss, 2009).

- Both T and B lymphocytes proliferate in response to mitogen stimulation. The level of response from lymphocytes extracted from healthy devils is similar to those from lymphocytes extracted from DFTD affected animals. Additionally, neutrophils from the peripheral blood show all the major features of the phagocytic process such as bacterial attachment and uptake of bacteria and oxidative burst (Kreiss *et al.*, 2008, Stewart *et al.*, 2008).

- Devils are also capable of mounting strong humoral responses against cellular antigens injected subcutaneously (Kreiss *et al.*, 2008, Kreiss *et al.*, 2009, Stewart *et al.*, 2008, Woods *et al.*, 2007).

DFTD is an allograft and foreign to the host. With a competent immune system, devils should be able to recognise and reject the tumour cells. However, sequence analyses showed that devils have extremely low levels of genetic diversity at the MHC genes (Siddle *et al.,* 2007b). Additionally, devils from eastern Tasmania showed weak responses to allogeneic mixed lymphocytes in culture (Woods *et al.,* 2007). This combined data suggested that DFTD and the host might be identical at the MHC loci allowing the tumour to spread without encountering any histocompatibility barriers (Siddle *et al.,* 2007a, Siddle *et al.,* 2007b).

Further studies of microsatellite markers and sequencing of MHC genes showed that devils from north-west Tasmania are genetically different from the eastern populations (Jones *et al.,* 2004, Siddle *et al.,* 2010). The studies revealed that devil MHC sequences fit into two distinct groups based on sequence similarity. DFTD cells and most devils have sequences from both groups. A small percentage of animals located in the north-west have a restricted MHC repertoire with only one group. The level of sequence variation between groups is significant. Consequently, animals with a restricted MHC repertoire were predicted to recognise DFTD and produce an immune response.

Unfortunately, monitoring and ecological studies of the disease in the north-west showed a different result. DFTD emerged in north-east Tasmania and rapidly spread throughout the eastern half of the island. The disease front has now reached the populations in the north-west and animals with restricted MHC repertoires have also been infected (Coupland and Anthony, 2007, Hamede *et al.*, 2012). Now it is evident that the lack of MHC diversity alone does not explain the transmission of DFTD.

Recent experimental transplantation of skin grafts between unrelated devils and mixed lymphocyte reactions (MLR) provided more evidence that the devils are capable of allogeneic rejection (Kreiss *et al.*, 2011a). Devils rejected the skin grafts regardless of MHC type and the strongest MLR responses occurred when mixing lymphocytes from eastern devils with lymphocytes from western animals.

Therefore, it is likely that DFTD evolved special adaptive mechanisms that allow allotransplantation of tumour cells without immune recognition. Downregulation of MHC expression and secretion of immune suppression molecules, as those discussed previously in CTVT, are very common in cancer (described in Section 1.5). MHC expression in DFTD has only been investigated at the genetic level. Further studies are required to determine whether MHC genes in DFTD are translated into proteins and functionally displayed in the surface of the cells. Immune suppression induced by the tumour needs to be explored as well.

Histopathology of DFTD

DFTD is characterized by primary tumours located around the mouth and face, which grow to in excess of 10 cm in diameter and often ulcerate (Figure 1.3). Tumours generally appear within the dermis or the sub mucosal connective tissue in the oral cavity. The neoplasms grow as a multi-nodular compact proliferation of pleomorphic round cells with a high nuclear to cytoplasm ratio. The nodular aggregates are well vascularized often within a thin fibrous pseudo-capsule and with high mitotic indices. In some cases, the cells aggregate as bundles, cords, or streams. Most of the tumours present central necrosis(Loh *et al.,* 2006a). An important aspect of DFTD tumours is the low evidence of lymphocyte infiltration (Loh *et al.,* 2006a). DFTD metastases are frequent in regional lymph nodes and other distant organs (Loh *et al.,* 2006a). Death occurs due to an inability to feed, secondary infections and organ failure due to the metastases (Pyecroft *et al.,* 2007).

Figure 1.3 Gross appearance of DFTD tumours in a wild Tasmanian devil. Tumours are generally located around the face and neck of the individual. Right panel, inferior view showing the ulcerated tumour.

1.3 Nature of devil facial tumour disease

1.3.1 Neuroectodermal origin

Studies of captive Tasmanian devils suggested that the species is prone to developing tumours (Canfield and Cunningham, 1993). However DFTD lesions do not resemble cancers previously described and determining its nature is critical for developing

management strategies for the disease (Loh *et al.*, 2006a). Initial immunohistological studies using routine diagnostic markers indicated that the tumour cells are of neuroectodermal origin and probably of neuroendocrine lineage (Loh *et al.,* 2006b). Further molecular analyses confirmed the neuroectodermal nature of DFTD but suggested that it is more likely to be related to the peripheral nerve lineage (Murchison *et al.,* 2010).

During vertebrate embryogenesis, the neuroectoderm extends caudally from the head region and forms the entire neural plate. Two key structures develop from the neural plate: *i*: the neural tube formed by the folding and fusion of the neural plate. This neural tube gives rise to the main cells types of the central nervous system (CNS), including neurons, astrocytes and oligodendrocytes (Kulesa *et al.*, 2004, Liu and Zhang, 2011). *ii:* the neural crest which derives from the dorsal lip of the neural tube. The neural crest cells formed in the neural crest are embryonic and transient progenitor cells that migrate extensively into the periphery. During and after migration these multipotent cells differentiate into a wide range of derivatives. Cells include neurons and glial cells from the peripheral nervous system (PNS), pigment cells in the skin (melanocytes); endocrine cells, and a variety of mesenchymal cell types (Figure 1.4) (Donoghue *et al.,* 2008, Dupin *et al.,* 2007, Joseph *et al.,* 2004, Le Douarin *et al.,* 2008).

Neuroectodermal tumours, also called neurological tumours, include all the neoplasms that derive from either the CNS or the PNS. The complexity and diversity of these tumours is enormous making classification difficult. In humans, most of the neurological tumours are of glial origin. This is related to the fact that glial cells retain the capacity to proliferate through life whereas neurons become post-mitotic after development (Zhu and Parada, 2002).

Characterization of neuroectodermal tumours (diagnosis and classification) is based on histopathology, clinical features, and molecular signatures of the predominant cell type in the tumour. Immunohistochemistry (IHC) is perhaps the most commonly method used for the diagnosis of human and animal neoplasms (Bodey, 2002, Heim-Hall and Yohe, 2008, Ramos-Vara *et al.*, 2008). The underlying principle of diagnostic IHC is that the immunophenotype of a tumour reflects the cell lineage in which the cancer originated (the concept of cell of origin in cancer was recently reviewed by Visvader,

2011). Thus, IHC benefits from the availability of a wide range of antibodies against specific markers of cell lineage and tissue type.

Loh et al. (2006b) used immunohistochemistry to analyse the expression of a wide range of diagnostic markers in DFTD. The pattern of protein expression suggested that DFTD was a neural crest-derived neoplasm. Positive markers included S100, neuron-specific enolase, synaptophysin, and chromogranin A, which are also consistent with cells of neuroendocrine origin. Further sequencing of the DFTD transcriptome confirmed the expression of neural crest cell markers in the tumour (Murchison *et al.,* 2010). Moreover, the study revealed a set of genes differentially expressed in DFTD tumours compared to normal tissue. Genes included transcription factors, structural genes, and receptors, which are involved in the differentiation of myelinating Schwann cells within the PNS. Therefore, the DFTD transcriptome suggested that DFTD is more likely to be a peripheral nerve tumour of Schwann cell origin.

As mentioned before neuroendocrine cells and glial cells of the PNS belong to two independent lineages that share a common origin in the neural crest. Therefore, it is feasible that DFTD express markers of both lineages. Confirmatory studies of the expression at the protein level of PNS markers will contribute to the understanding of the nature of DFTD.

1.3.2 Schwann cell development

The last decade produced important advances in the knowledge of the molecular mechanisms and pathways that control Schwann cell development, myelination and myelination maintenance within the PNS. Alteration of these processes has been linked to a variety of neuropathies and the formation of tumours of Schwann cell origin. These mechanisms provide new opportunities for our understanding of DFTD biology and behaviour.

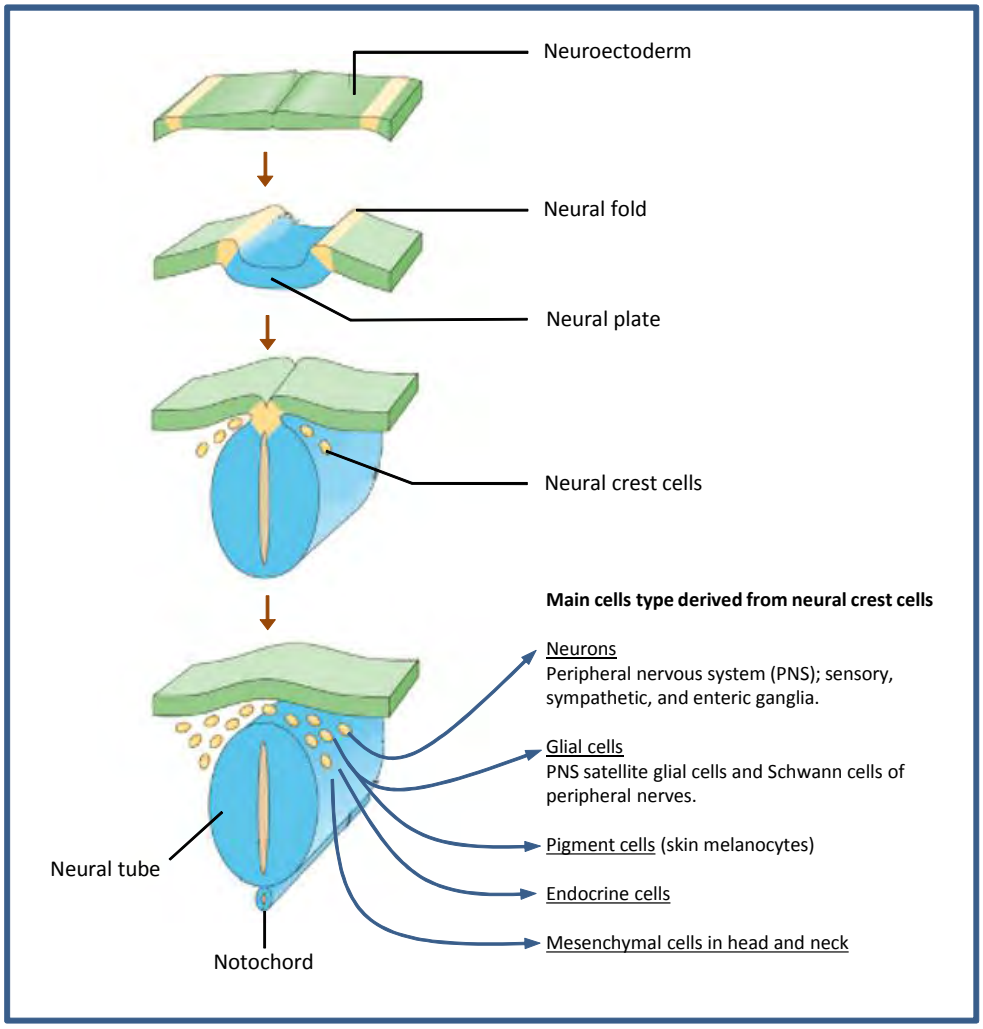

Figure 1.4 Development of the neural crest. The neural plate derived from the neuroectoderm folds and fuse to form the neural tube. Neural crest cells segregate from the tips of the fold and migrate in a lateral and ventral direction to generate a diverse type of cells. Adapted from Jessen and Mirsky (2005) and Dupin *et al.* (2007).

Key important findings on the research of the Schwann cell development include:

- Schwann cell development occurs through a series of transitional embryonic and postnatal phases.

During early embryonic development, neural crest cells (NCC) give rise to Schwann cell precursors (SCP) and then these generate the immature Schwann cells (ISC). At birth, either the immature Schwann cells differentiate into the myelinating or the non-myelinating mature Schwann cells (MSC) in the peripheral nerves. A complex network of extra and intra cellular signalling pathways regulates this process. Studies *in*

vitro and *in vivo* with rat and mouse models showed that at least two signals, neuroregulin-1 and endothelin, regulate the survival and differentiation of Schwann cells. Similarly, three transcription factors Sox-10, Oct-6 and Krox-20 appear to play key roles in the formation of the Schwann cell lineage. Krox-20 is also the major target of the signals that induce myelin differentiation (Jessen and Mirsky, 2002).

- Each individual stage of Schwann cell development (NCC, SCP, ISC and MSC) is clearly recognized by a range of phenotypic features including molecular markers, signalling responses and relationships to other cells and tissues.

For instance, Schwann cell precursors can be distinguished from neural crest cells by expression of brain fatty acid-binding protein (BFABP), protein zero (P0) and Cadherin -19. Differentiation of Schwann cell precursors to immature Schwann cells results in upregulation of markers such as GFAP and S100β as well as downregulation of other markers such as Cadherin-19 (Jessen and Mirsky, 2005). Additionally, it is likely that axonal signals control the differentiation of Schwann cell precursors into immature Schwann cells (Woodhoo and Sommer, 2008). The molecular phenotype of both immature and non-myelinating Schwann cells are relatively similar (Jessen and Mirsky, 2005). In contrast, the generation of myelinating Schwann cells involves more profound changes. Most of the antigens associated with immature Schwann cells are downregulated and there is an upregulation of a number of genes associated with control of myelination and formation of the myelination sheath (Jessen and Mirsky, 2005).

- The extracellular matrix (ECM) plays a critical role in controlling Schwann cell development (Bunge *et al.,* 1986, Chernousov *et al.,* 2008).

Various studies showed that the basal lamina that surrounds the Schwann cell-axon unit is crucial for Schwann cell differentiation and myelination. The interaction occurs through specific receptors on the surface of the Schwann cell. The best characterized receptors are the integrin receptors (Fernandez-Valle *et al.,* 1994). Distroglycan and proteoglycans can also mediate Schwann cell-ECM interactions (Nodari *et al.,* 2008). Proteins in the basal lamina that interact with these receptors include laminin and various types of collagen (Chernousov *et al.*, 2006, Sherman and Brophy, 2005). Other

studies suggested that it is the specific interactions between the receptors and the ECM-proteins that is critical for myelination and not the assembly of the basal lamina (Podratz *et al.,* 2001).

- Schwann cells are remarkably plastic and play an active role during nerve repair after injury.

Mature Schwann cells can both form and maintain the myelin sheath around axons and rapidly dedifferentiate following injury. During myelination, Schwann cells escape from the cell cycle and developmental death signalling and upregulate myelin proteins (Parkinson *et al.,* 2004). After injury, myelin proteins are downregulated and Schwann cells regain the potential to proliferate. These cells then re-differentiate and re-myelinate regenerated axons as a part of the repair process. A process of cross-inhibition between positive and active negative regulators controls the myelination and dedifferentiation stages. Recent studies by Mirsky et al. (2008) and Parkinson et al. (2008) have contributed to the understanding of these sophisticated mechanisms. During differentiation, immature Schwann cells receive axonal signals including neuregulin 1 (NRG) which upregulates the expression of transcription factors including NFκB, Oct-6, and Brn2. These transcription factors promote the promyelinating stage. Further upregulation of the transcription factor Krox-20 induces Schwann cells to express myelin-specific proteins and form the myelin sheath. Upon nerve injury, unknown signals induce a rapid upregulation of the transcription factors c-Jun and Sox-2. The former contributes to the downregulation of Krox-20 and the dedifferentiation of Schwann cells.

An important aspect of this process is that Schwann cells (but not precursors) can ensure their own survival in an autocrine way by secreting survival factors. Components of this circuit include insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB. This mechanism is important in maintaining the survival of Schwann cells in injured nerves even after axons have degenerated. Thus, Schwann cells can provide the essential support for axon regrowth (Meier *et al.,* 1999).

- Schwann cells may modulate local immune responses within the PNS.

Schwann cells can interact with the immune system by recognizing and presenting antigens, and may influence and terminate nerve inflammation by secreting cytokines (reviewed in Meyer zu Horste *et al.,* 2008).

1.3.3 The myelin sheath

The myelin sheath also called myelin is required for rapid transduction of nerve impulses and the normal functioning of the nervous system. The membrane of the Schwann cells (and oligodendrocytes in the CNS) wraps around the axon and forms this highly specialised cellular structure. The myelin is a lipid-rich multilayered membrane and includes a number of structural proteins many of which are only expressed by myelination cells. 20% to 25% of the PNS myelin consists of proteins, mainly myelin protein zero (MPZ). Other proteins include the myelin-associate glycoprotein (MAG), peripheral myelin protein 22 (PMP22), myelin basic protein (MBP), 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP), connexins (gap junction proteins), periaxin (PRX) and protein S100. Myelin also contains 70-80% lipid, including GalC, gangliosides and cholesterol (Kursula, 2008, Svaren and Meijer, 2008). Damages to the myelin sheath produced by disease and lesions can induce serious neurological conditions such as multiple sclerosis (Tzakos *et al.,* 2005). Genetic defects in the myelin proteins can also produce inherited peripheral myelinopathies. For instance, mutations in the PMP22, MPZ and periaxin genes cause one of the most common neuropathies, Charcot-Marie-Tooth disease (Hoyer *et al.*, 2011, Taioli *et al.*, 2011, Williams and Brophy, 2002).

1.3.4 Tumours of Schwann cell origin

Tumours of the PNS have been described in humans, dogs, cattle and other animals (Bundza *et al.*, 1986, Chijiwa *et al.*, 2004, Nielsen *et al.*, 2007, Schoniger and Summers, 2009, Schulman *et al.*, 2009). In humans, the benign neurofibroma and schwannoma are the two most common peripheral nerve sheath tumours (PNST). Malignant tumours include the malignant peripheral nerve sheath tumours (MPNST) which mostly derive from pre-existing benign neurofibromas. Less frequently, MPNST can arise in peripheral nerves without histological evidence of benign lesions. PNST may arise from

different type of cells including Schwann cells, perineurial cells, fibroblasts, and other cells comprising the nerve sheaths, however, evidence indicates that most neoplasms, benign and malignant, are in fact derived from Schwann cells (Carroll and Ratner, 2008, Dundr *et al.*, 2009, Gupta *et al.*, 2008, Zhu and Parada, 2002).

PNST can occur sporadically but the majority arise in individuals affected with neurofibromatosis type 1 (NF1) and type 2 (NF2). NF1 and NF2 are autosomal dominant tumour-suppressor gene syndromes in which affected individuals are predisposed to develop multiple tumours. Neurofibromas are the characteristic tumours developed in individuals affected with NF1. Some neurofibromas may undergo malignant transformation to MPNST, which are high-grade malignant tumours with patients having an average rate of survival of 5 years. NF2 affects approximately 1 in 35,000 individuals and patients often develop multiple schwannomas as well as a variety of clinical lesions (Ferner, 2010, Stemmer-Rachamimov *et al.*, 2004).

Most of the information into the initiation and progression of PNST tumours comes from studies in transgenic and knockout mouse models. Evidence provided by these models indicates that loss of NF1 or NF2 functionality in the Schwann cell lineage is sufficient to generate tumours. The loss of NF function also initiates a cascade of interactions with other cell types in the microenvironment and additional autonomous modifications which are also needed for tumour development and progression (Carroll and Ratner, 2008, Le *et al.*, 2011, Zheng *et al.*, 2008, Zhu *et al.*, 2002).

PNST nerves are histologically diverse and diagnosis still remains a challenge mainly due to the lack of specific immunohistochemical markers of neural differentiation (Sandberg, 2008). Although labelling for S100 protein shows some non-specific activity, it is the most commonly used marker to identify PNST of various types (Coindre, 2003, Wu and Montgomery, 2008). Other markers normally tested in PNST with different levels of reliability include MBP, NGFR, nestin and the neural crest transcription factor Sox 10 (Chijiwa *et al.*, 2004, Hoshi *et al.*, 1994, Nonaka *et al.*, 2008, Shimada *et al.*, 2007, Stasik and Tawfik, 2006). Therefore, diagnosis and classification of PNST requires correlation of IHC features with clinical and surgical data and in some cases electron microscopy.

1.4 The immune response to cancer

The immune response to cancer has been recognised for more than one hundred years and there is enormous research elucidating the molecular and cellular interactions between the tumour and the host immune system. The complexity of these interactions is so vast that there is still not consensus on the principles that explain why tumours develop and escape under the scrutiny of the immune system. A detailed review of our current knowledge of the immune response to cancer would be too long. Instead, I will focus on presenting the basic concepts that will contribute to the understanding of the more general cancer cell-immune system interactions. Another reason for this general review relates to the fact that our knowledge of the composition and function of the immune system of the Tasmanian devil is still limited. While the research in humans and other animal models is focusing on revealing the role of specific molecules, cell compartments and biochemical pathways, in the devil we are still trying to identify the main elements. Advances in devil research have been hindered by the lack of cross-reacting reagents. For instance, it is possible to identify T cells and B cells (Kreiss, 2009), but we still do not know the composition of the cell subpopulations. It is not yet possible to identify and characterise the cell types that play an important role such as natural killer cell, dendritic cells, regulatory T cells and macrophages. In practice, we are revisiting the elementary principles in an effort to understand the tumour-immune system interaction in the DFTD model.

1.4.1 Carcinogenesis and the cancer cell

Bubanovic and Najman (2005), specialists in comparative oncology and tumour immunology, defined tumour growth as a fundamental disorder in the regulation of cell division, growth, differentiation and cell socialization. In all vertebrates, neoplasia is a disease in which genetically altered cells escape from the normal cell cycle regulation and monitoring of the immune system. This results in a persistent, expanding or infiltrating growth without control of the architecture of the normal tissue.

It is generally accepted that one of the primary steps leading towards carcinogenesis in all vertebrates is the destabilization of the genome and loss of cell's ability to repair DNA damage. This is the consequence of series of mutations or translocations, and

comes largely as a result of the influence of chemical agents, viral infections, radiation and chronic inflammation (Bubanovic and Najman, 2005).

Tumours are transformations of normal cells in which growth control has become deregulated. As mentioned above, the molecular hallmark of carcinogenesis is genetic instability. This instability allows normal cells to evolve progressively to a neoplastic state and acquire the traits that enable them to become tumourigenic and malignant. Hanahan and Weinberg (2000) defined six principal biological capabilities (hallmarks) that cancer cells acquire during the development of tumours. These include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis Recently, due to the advances in the understanding of the tumourigenesis process, two more hallmarks were added: reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011). The authors also highlight the importance of the microenvironment in the development of tumours. They proposed tumours as a complex tissue composed of multiple cell types that interact with one another in an active way. The stromal cells, including fibroblasts, mesenchymal, endothelial and inflammatory cells, contribute to the development and expression of some of the cancer hallmarks. Table 1.1 presents a brief summary of these cancer traits and the underlying mechanisms.

1.4.2 The host defences against cancer

An important aspect deducted from the hallmarks of cancer presented in the previous section is the existence of multiple regulatory mechanisms (intra and extracellular) that prevent the emergence of cancer cells.

In this sense, the first line of defence from malignant cell alteration includes the mechanisms controlling cell cycle and DNA stability. A variety of intrinsic tumour-suppressor mechanisms including p53, sense the activity of oncogenes and initiate the programmed cell death machinery (apoptosis) or senescence. Similarly, cellular stress, injury, or lack of survival signals and alterations in mitochondria integrity trigger apoptosis. Cell-surface death receptors, such as tumour necrosis factor receptor (TNFR), can also activate cell death pathways (Vesely *et al.,* 2011). As seen in Table 1.1,

alternative cell death pathways such as necrosis and autophagy may also intervene in the transformation process. In summary, both senescence and apoptosis prevent the acquired capacity of cells to proliferate without environmental control and act as a barrier to the further development of neoplastic cells.

Table 1.1 The hallmarks of cancer. Capabilities of the cancer cells acquired during the development of tumours.

Cancer hallmark*	Mechanisms
Sustaining proliferative signalling	Chronic proliferation by deregulating the signals that control cell cycle and cell growth
Evading growth suppressors	Inactivation/circumvention of pathways controlled by tumour suppressors genes such as RB (retinoblastoma-associated) and p53 Abolishing the mechanisms of cell-to-cell contact inhibition Evasion of the anti-proliferative effects of TGF-β
Resisting cell death	Evasion or limited apoptosis by damage of suppressor sensors such as p53 or increasing the expression of anti-apoptotic regulators Deregulation of the process of autophagy Necrosis having the potential of being pro-inflammatory and tumour promoting
Enabling replicative immortality	Protecting the telomeres at the ends of the chromosomes which provides capability for unlimited proliferation
Inducing angiogenesis	Permanently activating and controlling the angiogenesis signalling
Activating invasion and metastasis	Developing alterations in the cell shape and the attachment to other cells and the extracellular matrix
Reprogramming of energy metabolism	Adjustment of energy metabolism in order to fuel cell growth and division
Evading immune destruction	Evasion of antitumor mechanisms**

* Reviewed in (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). ** These mechanisms are further described in the next section.

Extrinsic tumour-suppressor mechanisms include those that prevent cancer cells from invading and spreading to other tissues (Vesely *et al.,* 2011). One of these mechanisms involves adhesion molecules such as integrins, which are proteins that facilitate the anchorage of cells to other cells or the extracellular matrix (ECM). These molecules

also mediate cytosolic events that influence cell proliferation, survival and motility and therefore can control processes such as angiogenesis, tumour growth and metastases (Stupack, 2007).

Another extrinsic factor involves the immune mechanisms that limit the transformation or tumour growth. Swann (2007), proposed three primary roles of the immune system in the prevention of cancer. First, it can protect the host from virus-induced tumours by controlling viral infections. Second, the prompt elimination of pathogens aids in the resolution of inflammation that could favour tumourigenesis. Finally, the immune system can specifically identify and eliminate tumour cells based on the expression of tumour-associated antigens (TAAs). The immune system identifies transformed cells that have escaped cell-intrinsic tumour-suppressor mechanisms and eliminates them before they can establish malignancy. This process is referred as immune surveillance and will be discussed in more detail in the following section.

The concept of immunosurveillance

In the late 1950s, Burnet and Thomas formulated the hypothesis that the immune system can recognise and destroy nascent transformed cells (Burnet, 1957a, Burnet, 1957b). The theory proposed that mutated tumour cells have distinctive structures (tumour specific antigens) that can be recognised by cells of the immune system. The concept was supported by the rejection of transplanted tumours induced by chemical carcinogens or virus in syngeneic mice. Thus, the immune surveillance suggested that lymphocytes act as sentinels recognising and eliminating continuously arising, nascent transformed cells (Burnet, 1970).

Following its introduction, the cancer immunosurveillance concept was highly debated and challenged (reviewed in Dunn *et al.,* 2002). Initial studies in mice with induced immunodeficiencies showed inconsistent results in relation to the susceptibility of these mice to spontaneous or chemically induced tumours (Stutman, 1975). Furthermore, studies with athymic nude mice in the 1970s showed no statistical differences in the incidence of chemically induced tumours in the nude mice compared to the wild type (Stutman, 1979).

In the 1990's, studies with inbred mouse lines that carried targeted mutations affecting specific components of the immune system contributed to the renaissance of the cancer immunosurveillance theory. Not only did these studies support the importance of the immune system to control tumour formation but also suggested the involvement of both the innate and adaptive immune systems. Functional removal of NKT cells, αβ and γδ T cells, NK cells, IFN-γ or interleukin 12 (IL-12) all led to increased susceptibility of the host to tumours. Vesely et al. (2011) presented a comprehensive compilation and review of these studies.

The cancer immunoediting concept

The immunosurveillance concept proposed that the immune system plays a vital role in the development of tumours. The theory, however, does not explain why tumours still develop in individuals with a functional immune system. Studies from the last decade suggest that immunosurveillance only represents one dimension of the complex relationship between the immune system and cancer (Dunn *et al.*, 2004a). An important study by Shankaran et al. (2001) reported that immune deficient mice lacking either lymphocytes or IFN-γ functionality, developed more spontaneous neoplasms and were more susceptible to carcinogen-induced tumours compare to wild type mice. In addition, transplantation experiments showed that tumours formed in the absence of an intact immune system are more immunogenic than tumours developed in immunocompetent hosts. These results showed that the immune system functions as an effective extrinsic tumour-suppressor system but also leads to the immunoselection of tumour cells.

Dunn et al. (2002), proposed the new term "cancer immunoediting" to describe the dual host-protective and tumour-sculpting actions of the immunity in developing tumours. The tumour immunoediting concept, known as the "three Es model" involves three phases. The following description of the three phases was extracted from the reviews by Dunn *et al*. (2004b), Schreiber *et al*. (2011) and Swann and Smyth (2007).

Elimination. This phase is an update of the process described in the initial theory of tumour immunosurveillance. The innate and adaptive immune systems work together to detect and eliminate tumour cells that are developing because the intrinsic tumour

suppressor mechanisms failed. The immune system detects "danger signals" such a Type I IFN that activates dendritic cells and promote the induction of adaptive anti-tumour immune responses. Other signals include damage-associated molecular pattern molecules (DAMP) because they are released directly from dying tumour cells or from damage tissues. A third potential mechanism may involve stress ligands that are frequently expressed on the surface of tumour cells. These include RAE-1 and H60 in mice or MICA/B in humans. These ligands bind to activating receptors on innate immune cells, leading to the release of pro-inflammatory and immunomodulatory cytokines that facilitates the development of a tumour-specific adaptive immune response. In most systems, effective responses require the additional expression of tumour antigens capable of propagating the expansion of effector $CD4^+$ and $CD8^+$ T cells. If all tumour cells are cleared the elimination phase represents the endpoint of the cancer immunoediting process.

Equilibrium. Rare tumour cell variants may survive the elimination phase and enter the equilibrium phase. In this phase, the adaptive immune system prevents tumour cell outgrowth and sculpts the immunogenicity of the tumour cells. The equilibrium could extend through the life of the host and it may represent a second stable endpoint of cancer immunoediting. Equilibrium represents a type of tumour dormancy specifically controlled by the immune system. During this period, the tumour cells could also continue to evolve and accumulate further changes such as DNA mutations or changes in gene expression. As this process continues, the immune system applies a selective pressure by eliminating susceptible tumours. Studies with mouse models showed that only the adaptive immunity, specifically interleukin-12 (IL12), INF-γ, $CD4^+$ and $CD8^+$ T cells, are responsible for maintaining the tumour cells in equilibrium (Koebel *et al.,* 2007).

Escape. If the immune system fails to eliminate the tumour, the process results in the selection of tumour cell variants that are able to resist, avoid, or suppress the anti-tumour immune response, leading to the escape phase. The immune system is no longer able to contain tumour growth and tumour cells emerge as visible tumours. Progression from equilibrium to the escape phase can occur because: *i*, the tumour cell populations change in response to the immune system's editing functions; *ii,* the host immune

system changes in response to increased tumour-induced immunosuppression or *iii,* immune system deterioration.

Recent studies in mice with broad immunodeficiency or specific antigenic tolerance showed that that recognition of tumour-specific antigens by lymphocytes is critical for immunoediting against sarcomas (Dupage *et al.*, 2012). The study also found that primary sarcomas were edited to become less immunogenic through the selective outgrowth of cells that were able to escape T lymphocyte attack. Thus, loss of tumour antigen expression or presentation on major histocompatibility complex I was necessary and sufficient for this immunoediting process to occur. Section 1.5 discusses the immune escape mechanisms in more detail.

The theory of tumour immune complexity

Supporters of the cancer immunoediting concept acknowledge that immunosurveillance is a heterogeneous process requiring the actions of different immune effectors in a manner that is dependent on the tumour's cell type of origin, mechanisms of transformation, anatomical localization and mechanisms of immunologic recognition (Dunn *et al.,* 2002). This heterogeneity somehow explains the subtle differences on the results of the many independent groups that support the role of the immune system in the protection against cancer.

However, results from a recent study have challenged again this concept. Ciampricotti et at. (2011) investigated the functional role of the adaptive immune system as a regulator of spontaneous $HER2^{+}$ breast tumour genes and pulmonary metastasis formation using a mouse mammary tumour virus (MMTV)-NeuT mouse model. In this model, mammary carcinogenesis is induced by transgenic expression of the activated *HER2/neu* oncogene driven by the MMTV promotor. To investigate the modulatory role of the adaptive immune system, MMTV-NeuT mice were intercrossed with recombination-activating gene-2 homozygous null ($Rag\text{-}2^{-/-}$) mice which are deficient for mature T and B lymphocytes. They found that the absence of the adaptive immune system did not modified tumour progression. Similarly, the phenotype, behaviour (growth, multiplicity, and immunogenicity) and inflammatory microenvironment of primary breast tumours arising in the immune deficient mice were identical to those in

the immune proficient mice. Additionally, they demonstrated that pulmonary metastasis formation is not dependent on the adaptive system. Therefore, they suggested that spontaneous tumourigenesis and metastasis formation of $HER2^+$ breast cancer are not suppressed by the immunosurveillance mechanisms or promoted by the adaptive immune system.

Manjili (2011) has proposed a new model to explain the conflicting results that question the role of the immune system in the development of tumours. Manjili considers that the contradictory results imply the existence of different pathways, which may be complementary rather than conflicting. Thus, the model proposes that the tumour cells and the immune system interact in a complex network that is not simply "a one-way road". The different interactions between tumour cells and the immune system relates to the heterogeneous nature of tumours and their genetic instability. According to the model, dangerous clones of tumours elicit an immune response and may trigger tumour immunosurveillance and immunoediting mechanisms. Non-dangerous clones fail to elicit a response and may induce immune tolerance. The new extended model proposes 10 possible pathways used by the tumour cells to survive under the monitoring of the immune system.

1.5 Mechanism of immune evasion in cancer

Tumour cell escape can occur through many different mechanisms and involve the tumour, the tumour microenvironment and various components of the innate and adaptive immune systems. This section will focus on the mechanisms at the tumour level, as these are particularly relevant for the present research.

1.5.1 Generating an immunosuppressive tumour microenvironment

Cancer cells can develop an immunosuppressive state within the tumour microenvironment by various mechanisms:

- Producing immunosuppressive cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), galectin, indoleamine 2,3-dioxygenase (IDO) or interleukin-10 (IL10) (Zou, 2005). These factors are released

after activation of Stat3 and Braf signalling and can induce inhibition of both the innate and adaptive anti-tumour immunity (Drake *et al.,* 2006).

- Recruiting regulatory immune cells that function as the effectors of immunosuppression. Regulatory T cells (T_{reg} cells) and myeloid-derived suppressor cells (MDSC) are two major types of immunosuppressive leukocyte populations that play key roles in inhibiting host-protective antitumour responses. T_{reg} cells are $CD4^+$ that express CD25 and the transcription factor Foxp3. When stimulated they inhibit the function of tumour specific T lymphocytes by producing the immunosuppressive cytokines IL-10 and TGF-β. T_{reg} cells also express the negative co-stimulatory molecules CTLA-4, PD-1 and PD-L1. They also consume IL-2, a cytokine that is critical for maintaining CTL function. MDSC are a heterogeneous group of myeloid progenitor cells. These cells produce TGF-β that induce T_{reg} cells which inhibit lymphocyte function. MDC cells can sequestrate or deplete the amino acids arginine, tryptophan, or cysteine, which are required for T cell function. MDC cells can also block T cell receptors or chemokine receptors on tumour-specific T cells (Drake *et al.*, 2006, Zou, 2005).

1.5.2 Tumour cell modifications to evade immune detection and destruction

At the cellular level, tumour outgrowth is promoted by alterations that reduce immune recognition or increase resistance to the cytotoxic effects of immune cells. These alterations are produced by the combination of the genetic instability of the tumour cells and the process of immune selection. The result is the generation via a "Darwinian selection process" of poorly immune-genetic cell variants that become invisible to the immune system and acquire the capacity to grow progressively (Schreiber *et al.,* 2011).

Tumour cells can become resistant to cytotoxic effects through the induction of anti-apoptotic mechanisms. However, the reduction of the tumour immunogenicity is perhaps one of the most common and best-studied mechanisms of tumour evasion. The section below describes the roles of the MHC in the processes of antigen presentation and transplantation. This will follow by the presentation of the mechanisms by which tumours reduce immunogenicity.

Major histocompatibility complex (MHC) and antigen presentation

Major histocompatibility complex (MHC) molecules are the foundations of the self and non-self discrimination in vertebrates. The name of these highly polymorphic proteins relates to their function as being the major barrier for allogeneic transplantation (Kumanovics *et al.,* 2003). The MHC loci are grouped into three classes of genes called Class I (MHC-I), Class II (MHC-II) and Class III (MHC-III). The three classes are distinguished based on both structure and function of their encoded proteins. MHC-I molecules are expressed in virtually all cells and present cytosol-derived peptides to $CD8^+$ T cells. MHC-I molecules are also important for the immune regulatory activity of NK cells (Ljunggren and Karre, 1990). MHC-II molecules are expressed in antigen-presenting cells such as dendritic cells and typically present exogenous derived peptides to $CD4^+$ T cells. The MHC-III genes encode a variety of immune and non-immune system related molecules, most of which are not involved in antigen presentation, and include cytokines and components of the complement system (Belov *et al.,* 2006).

MHC-I molecules are heterotrimers consisting of a 45-kDa glycosylated transmembrane heavy chain, a soluble 12 kDa subunit beta-2 microglobulin (B2M) and an 8-10 residue peptide ligand (Germain and Margulies, 1993). In humans, the heavy chain is encoded by genes located within the MHC region in chromosome 6, whereas B2M is encoded in chromosome 17. MHC-I can divided into classical and non-classical molecules. The classical class I molecules are expressed on the surface of most mammalian cells with only few exceptions. Most glandular or squamous epithelia, as the surrounding connective tissue, express MHC-I antigens. However, the intensity of expression varies between different locations. For instance, skeletal, smooth muscle, gastric mucosa and the central and peripheral nervous system are weakly positive. The non-classical MHC-I have a much more limited degree of polymorphism and a more restricted tissue distribution and often perform other functions than antigen presentation (Garcia-Lora *et al.,* 2003).

MHC-I molecules bind peptide fragments of intracellular proteins and display them at the cell surface where they function to report the presence of virus or tumours to $CD8^+$ cells. These antigens peptides are generated from degraded endogenous proteins (tumour-associated antigens TAAs in the case of tumour cells) by the antigen presenting machinery. This process is known as antigen processing and requires four major steps: *i,* the generation of antigenic peptides by a large multicatalytic protease complex called proteasome and

other cytosolic proteases. *ii,* the transport of these peptides from the cytosol into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing-1 and -2 (TAP1 and TAP2). *iii,* the assembly of the peptides with MHC-I molecules. This depends on the association of the MHC-I heavy chain, first with B2M, and then with the peptide. The process involves components of the general glycoprotein quality control and MHC-I specific components including the chaperone tapasin (Zhang and Williams, 2006). i*v.* the exit of the tri-molecular complex (MHC-I heavy chain, B2M and peptide) from the ER through the Golgi secretory pathway and display on the cell surface for presentation to $CD8^+$ T cells (Solheim, 1999). The binding of high affinity peptide is crucial for the release and efficient transport of class I molecules from the ER – Golgi compartments and for class I stability on the cell surface at the physiological temperature (Donaldson and Williams, 2009). Any defect of these processes will lead to non-expression of MHC-I molecules on the cell surface (Garcia-Lora *et al.,* 2003).

The interaction of cells displaying MHC-I with the T cell receptor (TCR) on $CD8^+$ T cells triggers a cascade of T signalling events that ultimately lead to cell proliferation, cytokine production and lysis of the target cell. MHC-I antigens also regulate the lytic activity of NK cells which is related to their ability to kill target cells lacking MHC-I expression. Although NK cells do not express the TCR, the detection of targets by NK cells is mediated by two major families of receptor molecules belonging to the killer immunoglobulin-like receptor and to the C-type lectin superfamily (Garcia-Lora *et al.,* 2003).

Role of the major histocompatibility complex in transplantation

As mentioned before, MHC molecules are the foundations of the self and non-self discrimination in vertebrates and therefore are mainly responsible for graft rejection. T cells constitute the principal effector arm of allorecognition *in vivo* and *in vitro* (in mixed lymphocyte reactions). In contrast to the low frequency of T cells that recognise conventional antigens, as many as 10% of T cells respond to an alloantigen (Sherman and Chattopadhyay, 1993). The donor MHC antigens present in the allogeneic tissue are the main targets of the cellular immune response against the allograft. The recognition of mismatched donor histocompatibility antigens is the primary event that ultimately leads to allograft rejection (Sherman and Chattopadhyay, 1993).

Allorecognition occurs through two unique but not mutually exclusive pathways called direct and indirect pathways of antigen presentation. The direct pathway results from the recognition of intact foreign major histocompatibility molecules on the surface of donor cells. Indirect allorecognition occurs in the normal way by which the immune system normally recognises antigens. Donor histocompatibility molecules are internalised, processed, and presented as peptides by host antigen presenting cells (APC) (Game and Lechler, 2002). Both $CD4^+$ (which recognise donor MHC-II) and $CD8^+$ T cells (which recognise donor MHC-I) can mediate direct and indirect allorecognition (Bharat and Mohanakumar, 2007).

Additionally to MHC antigens, minor histocompatibility antigens can also activate the immune system against the allograft. These antigens are coded by non-MHC genes and are able of binding to both MHC-I and MHC-II molecules. In this way, these molecules can induce $CD4^+$ and $CD8^+$ T cell responses. Minor histocompatibility antigens generally relate to a polymorphism of any protein between donor and recipient. These proteins can be processed and presented on self MHC and potentially elicit graft rejection (Ingulli, 2010).

In addition to antigen recognition, the inflammation and stress produced by the organ transplantation can activate the recipient immune response. Studies in mice models showed that induction of several neutrophil and macrophage chemo-attractants were required for optimal recruitment of T cells into the graft (Morita *et al.,* 2001). Similarly, Bharat et al., (2007) demonstrated that the early release of cytokines plays a crucial role in the development of alloimmunity and human lung allograft rejection.

In summary, the rejection of allografts is the result of complex coordination between both the innate and adaptive immune system. T cells are central to the process as they are able to recognise donor derived antigens presented by MHC molecules. Once the T cells become activated, they undergo clonal expansion and differentiation into effector cells. Then these cells migrate into the graft where they promote tissue destruction. In addition, help B cells produce alloantibodies increasing the response (Ingulli, 2010).

Expression of MHC-I in the nervous system

The ability of cells within the CNS and PNS to express MHC-I molecules has been debated for many years. Contributing to this debate is the widely accepted concept that the brain is an immunologically privileged organ and therefore it was considered that neurons would

not express MHC-I genes. However, research is producing evidence that neurons in the CNS and the Schwann cells in the PNS express MHC-I molecules.

Different studies have provided strong evidence that neurons and Schwann cells increase the levels MHC-I under special conditions or treatments. For instance, neurons express high levels of MHC-I protein after axotomy (Maehlen *et al.*, 1988), nerve injury (Cullheim and Thams, 2010), viral infection (Chevalier *et al.*, 2011, Redwine *et al.*, 2001) and exposure to cytokines (Fujimaki *et al.*, 1996, Neumann *et al.*, 1997). Similarly, Schwann cells express MHC-I molecules under pro-inflammatory conditions (Armati *et al.*, 1990, Lilje and Armati, 1997) and in neuropathies such as the Guillain–Barré syndrome (GBS) (Wanschitz *et al.*, 2003).

Under normal conditions the *in vivo* expression of MHC-I protein in the nervous system is still controversial. Boulanger *et al*. (2004) argued that the inconsistences in the detection of MHC-I expression in neurons of the CNS could be due to technical issues. These include the lack of specific antibodies for use in fixed tissues compared to flow cytometry analyses performed in live cells, lack of antibodies cross-reacting to the whole population of MHC-I molecules, and biological factors including the development stage and subset of neurons analysed. Despite the controversial nature of the data in this area, there is still a consensus that MHC-I expression in neurons is low compared with the levels observed in other tissues such as spleen (Corriveau *et al.*, 1998) and the endothelial cells that line CNS blood vessels (Boulanger and Shatz, 2004). Like the CNS, many reports of in vivo MHC-I expression in Schwann cells relate to neuropathies and exposure to cytokines (Lilje and Armati, 1997). The data under normal conditions is more complex. Flow cytometry studies of mouse Schwann cells showed that the cells express MHC-I at basal conditions (Meyer zu Horste *et al.*, 2010), while immunohistochemistry studies did not found expression in myelin sheaths compared to blood vessels and endoneurial macrophages (Wanschitz *et al.*, 2003). Thus, the presence and changes of MHC-I expression in the peripheral nerve system is not completely understood.

Loss of tumour antigenicity

Changes in the expression and/or function of the MHC-I antigens is one of the best documented mechanisms of tumour immune evasion in human cancers. MHC-I alterations occur through different mechanisms that include downregulation or total loss of MHC-I

molecules or other molecules of the antigen processing machinery (Seliger *et al.,* 2002). These defects contribute to the immune evasion via resistance of tumour cells to killing by activated immune cells (Dunn *et al.,* 2002). Studies revealed that 63% of melanoma, 88% of breast carcinoma, 77% of laryngeal, 74% of colorectal, 82% of prostate, and 77% of bladder tumours have various types of abnormal MHC-I expression (reviewed in Aptsiauri *et al.,* 2007).

The molecular mechanisms leading to alteration in MHC-I expression are not well understood and can occur at any step required for MHC-I synthesis, assembly, transport or expression at the cell surface (Mendez *et al.,* 2008). Additionally, these defects can occur at the genetic, epigenetic, transcriptional and post-transcriptional levels. Garrido et al. (2010), classified the MHC alterations into two main groups: reversible defects (regulatory or "soft") including those abnormalities that can be recovered with cytokine treatment, and irreversible defects (structural or "hard") including the structural abnormalities that cannot be reversed (Table 1.2). Both reversible and irreversible structural defects have been described in tumour cell lines, animal models and in primary tumours and metastases obtained from cancer patients (Garrido *et al.,* 2010).

The understanding of the processes of loss of tumour immunogenicity has been useful for the evaluation of immune-therapeutic approaches into the clinic and a number of strategies are investigated in clinical trials. For instance, studies have shown that by silencing HER2, an oncogene normally expressed in breast and other cancers, tumour cell lines increased the expression of MHC-I molecules (Choudhury *et al.,* 2004). Additionally, experimental models have shown that HER2 vaccines can induce tumour protection in vivo (Scardino *et al.,* 2007). The general assumption of these studies is that vaccination methods that enhance the avidity of MHC-I restricted T cells can overcome the relatively poor antigen presentation in tumours (Ladjemi *et al.,* 2010).

The interaction between the tumour cells and the immune cells is a highly complex system. This is reflected in the variability of the responses on cancer patients to immunotherapy. There is a unanimous call to incorporate a more holistic approach for the development of immune therapies. Approaches should consider the molecular make-up of the tumour as well as the tumour microenvironment. The aim is to combine tumour therapies that trigger a multifaceted immune response involving humoral, cellular and innate immunity (Garrido *et al.*, 2010, Poschke *et al.*, 2011, Zou, 2005).

Table 1.2 Molecular defects leading to MHC-I altered expression in tumours.

Irreversible structural defects	
MHC gene	Loss of heterozygosity (haplotype loss) Mutations of MHC-I heavy chain (allelic loss)
B2M gene	Loss of heterozygosity Mutations/deletions
IFN transduction pathway	Blockade of the Jack-STAT pathway
Reversible regulatory defects	
Transcriptional downregulation	Coordinated downregulation of heavy chain, β2m and APM molecules
Hypermethylation	MHC-I genes
Oncogene activation (c-myc, HER-2/neu, oncogenic adenovirus 12)	Downregulation of MHC-I and APM genes Inhibition of the post-transcriptional processing of MHC-I mRNA

APM: antigen processing machinery (adapted from Garrido *et al.,* 2010).

1.6 Tumour antigens and immunotherapy

1.6.1 Tumour antigens

Section 1.4 introduced the concepts of immune surveillance and cancer immunoediting. Evidence for this response is demonstrated in part by the identification of antibodies against a number of intracellular and surface antigens detectable in sera from patients with different cancer types (Gnjatic *et al.,* 2003, Jager *et al.,* 2000). Molecular studies revealed that tumour-associated antigens (TAAs) are the consequence of genetic and epigenetic alterations in cancer cells or the products of genes encoding viral proteins (Pardoll, 2003, Schreiber *et al.*, 2011). The reason for spontaneous humoral responses in cancer patients are not known but may include an overabundance of antigen or its immunogenicity in the malignant setting (Vesely *et al.,* 2011).

MAGE-1 (melanoma antigen) was the first gene reported to code for human tumour antigen recognised by autologous T cells (van der Bruggen *et al.,* 1991). Different proteomic and immunologic approaches have revealed hundreds of different antigens from several human tumours (reviewed in Novellino *et al.,* 2005, Reuschenbach *et al.,* 2009). Initially, tumour antigens were classified as tumour-specific antigens (TSAs) including those antigens expressed only by tumour cells, and tumour-associated

antigens (TAAs) including the mutated counterparts of proteins expressed in normal cells. The current classification only includes TAAs, which are divided into shared and unique TAAs.

Reuschenbach at al. (2009) performed a systematic literature review of TAAs eliciting humoral responses in cancer patients and found more than 100 antigens. The majority of these antigens were related to overexpressed or mutated proteins. Most of the antigens represented cytoplasmic proteins (42%), 26% were expressed predominantly in the nucleus (transcription factors, cell cycle regulators), 21% were membrane bound proteins (receptors, cell adhesion proteins) and 10% were extracellular proteins (extracellular matrix proteins, secreted proteins). The most frequently analysed antigens were p53, MUC1, NY-ESO-1, c-myc, survivin, p62, cyclin B1 and HER-2/neu. Antibodies against these antigens were frequently detected in cancer patients, while for most of antigens, antibodies were rarely found in healthy individuals. The most frequently analysed tumour sites were breast, colorectal, liver and lung.

A similar review listed all the available TAAs recognised by T cells (either $CD8^+$ or $CD4^+$) (Novellino *et al.,* 2005). These antigens were classified into four different groups. The best characterized are shared antigens called *cancer-testis (CT) antigens* because of their expression in different human tumours and, among normal tissues in spermatocytes of testis and occasionally placenta. CT antigens result from the reactivation of genes that are silent in normal tissues. They include the MAGE, BAGE and GAGE gene families. The second group are *differentiation antigens* shared between tumour and normal tissue from which the tumour arose. These TAAs are mostly found in melanomas and normal melanocytes and are normally involved in the biosynthesis of melanin. The third group include widely occurring and *overexpressed TAAs*. These antigens are present in histologically different types of tumours as well as in many normal tissues, generally with low level of expression. It is possible that these overexpressed antigens trigger an anti-cancer response by breaking previously established tolerance. Among these TAAs are the anti-apoptotic proteins livin and survivin, hTERT and the tumour suppressor protein p53. The final group include *unique TAAs* that arise from point mutations of normal genes such as β-Catenin, caspase-8 and

CDK-4. Unique TAAs are considered the most specific targets for cancer immunotherapy in humans.

1.6.2 Identification of TAAs

Immunoproteomics, which defines the subset of proteins involved in the immune response, is the most common method to identify humoral responses in cancer patients. This method allows the individual screening of sera from a large number of patients. Immunoproteomics also permits the determination of relevant autoantigens eliciting immune responses and the distinction of isoforms. More importantly, the technique is useful for the detection of antibodies against post-translational modifications or specific targets (Mou *et al.,* 2009). Desmetz et al. (2009), reviewed the advantages and drawbacks of the current proteomic approaches used for the study of humoral responses. Table 1.3 presents a summary of this information.

1.6.3 Cancer immunotherapy using TAAs

Research into the identification of TAAs that elicit immune responses is constantly increasing. TAAs have been useful for screening, prognosis and diagnosis in different human cancers. For instance, higher titters of HER-2/neu are detected in early stages of breast cancers (Disis *et al.,* 1997). Overexpression of MUC1 and HER-2/neu are also associated with aggressive behaviour of the tumour and poor disease outcome (Menard *et al.,* 2004, Yonezawa *et al.,* 2008). NY-ESO has been found frequently in advanced tumour stages of oesophageal cancer (Akcakanat *et al.,* 2004). Poor survival has been demonstrate for p53-antibody positive patients in various type of cancers (Abendstein *et al.,* 2000, Lai *et al.,* 1998, Tang *et al.,* 2001).

Table 1.3 Proteomics techniques for the screening and validation of tumour associated antigens.

Technique	Description	Sensitivity	Throughput	Advantages	Disadvantages
SERPA	Tumour proteins are separated by 2-DE followed by identification by MS	Moderate	Moderate	Identification of TAAs *in vivo* Identification of post-translational modifications and isoforms	Identification of low-abundance TAAs limited Identification of transmembrane TAAs limited
SEREX	Tumour proteins are expressed by phage bank	Moderate	Moderate	Identification of TAAs *in vivo*	TAAs' post-translational modifications impossible to detect if using phages Complex False-positive
Protein array	Purified proteins or phage clones (each expressing a specific tumour protein) or fractioned tumour cell lysates are printed on slides	High	High	High throughput allowing autoantibody profiling Laboratory efficient	Expensive Requires protein synthesis at high quality
Antibody array	High quality antibodies are printed on slides and are incubated with whole proteins extracts	High	High	High throughput allowing autoantibody profiling TAAs are immobilized into their native configuration Direct information is provided on the TAAs	Expensive Require highly specific and affine antibodies
ELISA	Known antigens are coated on 96-well plates	Highest	Moderate	Robust Well-established use in clinical assays	Requires well-characterized protein and antibody for detection and extensive validation

SERPA, Serological proteome analysis; SEREX: Serological identification of recombinant expressed clones. 2DE, Two-dimensional electrophoresis; MS, mass spectrometry. Adapted from Desmetz *et al.* (2009).

The identification of TAAs has also been useful for the development of immunotherapy strategies. Current approaches can be passive or active. Antibody based immunotherapy (passive) is limited as it can target only cell surface or secreted proteins. However is has been effective in breast carcinoma with antibodies against the transmembrane protein ERB2 (HER2) (Baselga and Swain, 2009) and the T-lymphocyte antigen-4 (CTLA-4) in melanoma (Eggermont *et al.,* 2010). On the other hand, active therapy has mainly focused on strategies that efficiently activate cytotoxic T lymphocytes (CTL). This is because CTL can recognise antigenic peptides derived from endogenously expressed proteins. Thus, CTL can recognise potentially all tumour-associated antigens (Hirohashi *et al.,* 2009). Additionally, CTL are the only cell type that can directly kill target cells in a highly and efficient manner (Poschke *et al.,* 2011).

There are numerous cancer vaccine clinical trials performed with vaccines based on peptides derived from known TAAs (Table 1.4). Although the immunization trials induce a high frequency of specific T cells, the clinical outcomes have been limited (Rosenberg *et al.*, 2004). Several reasons may account for the lack of effectiveness in these trials: *i,* tumour evasion from immune recognition. This include selection of resistant tumour variants, antigen loss and induced immune tolerance induced by shared TAAs; *ii*, inefficient induction of high affinity adaptive immunity and *iii,* tumour-induced immunosuppression (Buonaguro *et al.,* 2011).

As mentioned before it has become apparent that successful immunotherapy of tumours will require a better understanding of the natural relationship between the tumour and the immune system. The new strategies aim to use a combination of approaches that target each of the key elements in anti-tumour immunity. The objective is to efficiently trigger both the innate and adaptive (humoral and cellular) immune system. This will elicit an adequate level of effector cells and establish immunological memory (Buonaguro *et al.*, 2011, Reuschenbach *et al.*, 2009).

Some of the approaches already adopted include immunisation with whole tumour proteins containing multiple and relevant antigenic epitopes that increase the chance of polyvalent B and T cell activation (Jager *et al.*, 2001). Similarly, cytokines used as adjuvants not only increased T cell activation but also helped to control regulatory T cells (which induce T cell tolerance) (Hara *et al.,* 2000, Litzinger *et al.,* 2007).

Furthermore, dendritic cells loaded with peptides or proteins *in vitro*, or transduced with the relevant genes represent one of the most promising strategies (Osada *et al.,* 2006).

Table 1.4 TAAs-based cancer vaccines for most relevant tumours.

Tumour	Antigen (# of clinical trials registered)	Phase
Acute myelocytic leukaemia	WT1 (5), PR (5)	I/II
Breast	E75 (2); p53 (2); HER-2/neu (9)	I/II
Colorectal	Ras (5); CEA (4)	I/II
Lung	URCL10 (6); ras (4); HER-2 (2); VEGFR1 and 2 (3); mutant p53 (2)	I/II
Melanoma	MAGE (13); gp100 (54); MART-1 (36); Tyrosinase (32); NY-ESO-1 (4)	I/II
Ovarian	p53 (4); NY-ES0-1 (3); HER-2 (3)	I/II
Uterine	HPV16 E7 (4); Survivin (1); mutant p53 (1)	I/II
Pancreas	ras (4); VEGFR1 and 2 (3); MUC-1 (1); Survivin (1)	I/II

Adapted from Buonaguro *et al.* (2011).

Our group is conducting an immunisation program in order to trigger an immune response against devil facial tumour cells. Preliminary immunisation trials with non-viable DFTD cell lines revealed that Tasmanian devils are able to produce a humoral (antibody) response against the cancer cells. Based on the human model, the identification of the DFTD antigens eliciting these humoral responses it is significant for the development of immune targeted therapies or a vaccination approach that would contribute to the conservation of the species.

1.7 Summary

The review presented in the previous pages highlighted the major advances in our understanding of the interactions between cancer cells and the immune system. Potentially, one of the most important progresses is the accepted scientific consensus on the participation of the immune system in the control of tumour development. Evidence for this is the inclusion of *immune evasion* as a "hallmark" of cancer. It is also accepted that the immune system plays a paradoxical role in this process. It participates in the

control of emerging cancer cells but can also promote tumour progression and development. Research from different groups shows that the interaction between cancer cell-immune system is multifactorial and highly complex. The trend is to change the "one single way" approach and adopt a more holistic one that also includes the tumour microenvironment as an important factor mediating both tumour initiation and progression.

Similarly, substantial efforts have been made to understand the mechanisms of transmission of the fatal cancer that is threatening the Tasmanian devil with extinction. Karyotyping and genetic analysis confirmed allotransplantation of DFTD cancer cells as the means of transmission. Sequencing analyses indicate that Tasmanian devils have limited diversity at the major histocompatibility complex (MHC) loci and this was postulated as the main reason why devils do not reject the cancer cells. Recent evidence showed that the limited MHC diversity is enough to cause rejection of allogeneic skin grafts and adequate to mount high responses in mixed lymphocyte reactions. Therefore, more research is needed to understand the nature of the tumour and the lack of immune response to the transfer of allogeneic cancer cells.

Investigation of the immune system of the Tasmanian devil and DFTD has been hindered for the lack of cross-reacting reagents. However, the recent sequencing of the devil and DFTD genome and transcriptome has facilitated and opened new opportunities of study.

Using these new tools, the present research focuses on three specific areas of interest. *i*, the understanding of the nature of DFTD and the exploration of biological markers that could be useful for diagnosis of the disease. *ii,* the understanding of the mechanisms that favour DFTD immune evasion and *iii,* identification of DFTD tumour associated antigens.

1.8 Research aims

Aim one: To determine the nature of DFTD and identify tumour markers that could be used for the development of diagnostic tools.

DFTD is a transmissible fatal cancer. Our knowledge about the biology of this tumour and the immune response of Tasmanian devils is very limited. Understanding the nature of the tumour could lead to the discovery of specific diagnostic markers for the disease and will provide insights into the mechanisms used by DFTD to escape the devil's immune system.

Chapter 2 presents an analysis of the histogenesis of DFTD based on previous immunohistochemistry studies and the devil transcriptome.

Chapter 3 evaluates the utility of the expression of proteins in DFTD as diagnostic markers for the disease.

Aim two: To evaluate MHC-I expression in DFTD as a mechanism used to escape the immune system.

Immunisation trials with DFTD cells showed that some Tasmanian devils can produce an immune response against DFTD. However, this immune response is unlikely to be protective. In order to produce an effective response, endogenous antigens (such as those produced by cancer cells) need to be transported and displayed at the surface of the cell. MHC-I molecules are essential components of this process. Genetics studies indicate that DFTD tumour cells express MHC-I molecules. However, these molecules have not been detected at the protein level.

Chapter 4 presents and evaluation of the expression, at the protein level, of MHC-I molecules in DFTD.

Aim three: To identify the DFTD tumour specific antigens that bind to antibodies in DFTD immunised devils.

Chapter 5 presents an analysis of the reactivity of the serum of immunised Tasmanian devils in order to identify DFTD tumour associated antigens.

2 Histogenesis of devil facial tumour disease

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2.1 Introduction

Devil facial tumour disease (DFTD) emerged around 1996 in north-east Tasmania. The disease is a contagious cancer that has already spread in more than 60% of the geographical extension of the species. Some local devil populations have declined by more than 80% (Hawkins *et al.*, 2006). Cytogenetic and molecular studies confirmed that the infectious agent is the cancer cell itself (Pearse and Swift, 2006, Siddle *et al.*, 2007a). However, our knowledge about the nature and biology of this tumour is limited.

In this context, the first systematic study of cell type and classification of DFTD was undertaken by Loh and colleagues (2006a) using standard pathological techniques such as cytology, histology and transmission electronic microscopy. Their results described the tumour masses as a malignant undifferentiated soft tissue neoplasm. Further immunohistochemistry analysis of DFTD tumour cells conducted by the same group concluded that the neoplasm was of neuroectodermal origin and consistent with a malignant neuroendocrine tumour (Loh *et al*., 2006b).

Recently, Murchison and collaborators performed a large-scale genetic analysis of DFTD tumours and devil tissues that included deep sequencing of the tumour transcriptome. This study revealed that DFTD expresses a set of genes related to the myelination pathway in the peripheral nervous system (PNS). Components of this molecular network included transcription factors (SOX10, SOX2, POU5F1, and Jun) and structural myelin genes such as protein zero (MPZ), periaxin (PRX), myelin basic protein (MBP) and peripheral myelin protein 22 (PMP22) (Murchison *et al.*, 2010).

The genetic studies on the transcriptome support the neuroectodermal origin of DFTD. However, although both the myelinating cells in the PNS and neuroendocrine cells derive from the neural crest, they develop from two different cellular lineages. Therefore, confirmation of the protein expression of the peripheral nerve markers is required to clarify the nature of the tumour. To achieve this, this chapter examined the expression of myelin and other neuronal proteins on tumour tissues using immunohistochemical techniques. Additionally, the expression of PNS markers in DFTD cell lines was evaluated using flow cytometry. Results of this study have been published in Murchison *et al*., (2010) and Tovar *et al*., (2011).

2.2 Methods

2.2.1 Tissue samples

A total of 20 primary DFTD tumours and 10 DFTD metastases were used in this study. The samples were supplied by the tissue bank held at the Menzies Research Institute Tasmania, University of Tasmania, and the Mount Pleasant Laboratories from the Tasmanian Department of Primary Industry, Parks, Water and Environment (DPIPWE).

2.2.2 Histology and Immunohistochemistry

Three-micrometre thick paraffin sections were prepared from DFTD tumour and normal devil tissues previously fixed in 10% buffered formalin and placed onto 3-aminotriethoxysilane-coated slides. Standard hematoxylin and eosin (H&E) staining was performed at the Anatomical Pathology Department of the Royal Hobart Hospital. For immunohistochemistry, sections were initially deparaffinised in a histology oven at 60 °C for 15 minutes followed by two five minutes washes in xylene, then rehydrated through successive graded ethanol solutions, and washed for five minutes in distilled water. Heat-induced antigen retrieval was performed in citrate buffer, pH 6 (Dako, Carpinteria, CA) using an electric pressure cooker at medium heat for 10 minutes, followed by a 20 minutes cooling period at room temperature. A complete list of reagents is presented in Appendix 2.

The slides were preincubated with serum-free blocking solution (Dako) for 30 minutes and then incubated with the primary antibody for 1 hour (see Table 2.1 for a complete list of antibodies). Endogenous peroxidase activity was then quenched by treating the slides in 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 minutes at room temperature. Signal detection was carried out using the anti-mouse (K4007) or anti-rabbit (K4003) Dako EnVision+ System, HRP, (Dako) and the DAB Substrate-Chromogen system (Dako) which yields a brown reaction end-product at the site of the target antigen (positive staining). The sections were counterstained with hematoxylin for 40 seconds and mounted.

The specificity of the primary antibodies was validated with recognized positive controls using appropriate normal devil tissues and negative controls by omitting the primary antibody for each immunohistochemistry run. Peripheral nerves bundles,

usually located in tissues adjacent to tumour nests, were used as a positive control for MBP, NGFR, NSE, PMP22, periaxin and S100; spinal cord was used as control for nestin and pituitary gland for CGA.

2.2.3 Interpretation of protein expression in tissues

A light microscope (Olympus-BX50) coupled with a camera (Leica-DFC320) was used for visualization and acquisition of the images. Immunoreactivity (i.e. protein expression) was considered positive when more than 10% of DFTD tumour cells within the tissue section were labelled with the antibody. Labelling reactions were analyzed semi-quantitatively by the principal author and a trained veterinary pathologist.

2.2.4 Flow cytometry

A DFTD cell line (C5065) was provided by the Mount Pleasant Laboratories from the DPIPWE. The cells were incubated to semi-confluence at 37 °C and 5% CO_2 in T25 cm^2 culture flasks in RPMI medium (RPMI 1640, Gibco, Auckland, New Zealand), 10% foetal calf serum (FCS), 2 mM of L-glutamine (Sigma, St Louis, MO) and 40 mg/ml of gentamicin (Pfizer, Sydney, Australia). Cell surface or intracellular expression of eight neuronal markers was tested in non-permeabilized or permeabilized cells respectively. For cell surface labelling, approximately 5×10^6 cells (number estimated using a Neubauer chamber) were transferred to microcentrifuge tubes and resuspended in 200 µl of PBS + 10% FCS (PBS-FCS) containing the primary antibody at the recommended concentration (Table 3.2). The tubes were mixed gently and incubated for 30 minutes on ice. The cells were washed three times with PBS-FCS by centrifugation at 10000 × *g* for 30 seconds. The final pellet was resuspended in 200 µl of PBS-FCS containing the secondary antibody goat anti-rabbit or goat anti-mouse Alexa Fluor 488 (Invitrogen). The cells were incubated for 30 minutes on ice and then washed 3 times as indicated above. Following the final wash, the cells were resuspended in 200 µl of PBS-FCS and transferred to flow cytometry tubes. For intracellular labelling, 5×10^6 DFTD cells were first permeabilized with Fix and Perm Cell Permeabilization Reagents GAS003 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Labelling was then performed as described above but replacing the PBS with the washing buffer provided with the kit. Isotype controls and negative controls experiments (by omitting the primary antibody) were run in parallel for both permeabilized and non-

permeabilized cells. Fluorescence detection was performed in a BD FACS Canto II flow cytometer and analysed using BD CellQuest Pro v5.2.1 software.

Table 2.1 List of antibodies tested by immunohistochemistry (IHC) and flow cytometry (FC)

Antibody	Official symbol (aliases)*	Species	IHC/FC Concentration	Company/Catalogue
Chromogranin A	CHGA (CGA)	Mouse IgG2b	1/50 - IHC	ABR Affinity Bioreagents/ MA1-37447
Myelin basic protein	MBP	Mouse IgG2b	1/400 - IHC	ABR Affinity Bioreagents/ MA1-10837
Nerve growth factor receptor	NGFR (p75NTR)	Rabbit IgG	1/100 - IHC 1/500 - FC	Abcam/ab8874
Nestin	NES	Mouse IgG1	1/250 - IH 1/200 - FC	BD Bioscience/611658
Neuron specific enolase/ enolase 2 gamma	ENO2 (NSE)	Mouse IgG1	1/50 - IHC	AbD serotec/MCA1764T
Periaxin	PRX	Rabbit IgG	1/300 - IHC 1/500 - FC	Sigma/HPA 001868
Peripheral myelin protein 22	PMP22	Rabbit IgG	1/100 - IHC 1/500 - FC	Abcam/ab61220
S100 calcium binding protein	S100	Rabbit IgG	1/300 - IHC	Dako/Z 0311
Myelin protein zero	MPZ	Rabbit IgG	1/500 - FC	Abbiotec 250762
Alexa Fluor 488 goat anti-rabbit IgG	-	Goat	1/1000 IF	Invitrogen/A11034
Alexa Fluor 488 goat anti-mouse IgG	-	Goat	1/1000 IF	Invitrogen/A11029

* Official symbol according to the HUGO Gene Nomenclature Committee – HGNC.

2.3 Results

2.3.1 Histological features of DFTD tumours

The histological appearance of DFTD tumours was identical to previous descriptions in both primary tumours and metastases (Loh *et al.*, 2006a). Briefly, tumour cells formed large nodular aggregates, often within a thin, fibrous pseudo-capsule and composed predominantly of neoplastic cells supported by minimal fibrovascular stroma. Cells could also be arranged in bundles, cords or streams separated by fine strands of collagen fibres. Cells were polygonal to round with a high nuclear to cytoplasm ratio (Figure 2.1.A-B). Mitotic figures were commonly observed with up to seven figures per high power field (40X) in some cases. Most of the large tumour masses contained central areas of necrosis. Inflammatory cells were rare and when present were mostly confined to the periphery of the tumour.

2.3.2 Protein expression of myelin and neuronal markers in DFTD tumours

This study evaluated the protein expression of the structural myelin proteins periaxin (PRX), peripheral protein myelin 22 (PMP22), and myelin basic protein (MBP). Peripheral neuronal markers included protein S100, nestin (NES) and nerve growth factor receptor (NGFR). Additionally, the neuroendocrine markers neuron-specific enolase (NSE) and chromogranin A (CGA) were also examined. Expression was considered positive for samples that showed immunolabelling in more than 10% of the tumour cells. Average labelling intensity was considered weak, moderate, or strong compared to that of the positive control (Table 2.2).

Periaxin, a protein that participates in the membrane-protein interactions that are required to stabilize the mature myelin sheath in peripheral nerves, was strongly expressed in 100% of DFTD primary tumours (n = 20) and 100% of DFTD metastases (n=10). Peripheral nerves surrounding the tumours were also clearly identified by their strong and specific immunoreactivity for periaxin (Figure 2.1.C-D).

Table 2.2 Expression of myelin and neuronal markers in primary DFTD tumours and metastases

Sample	Marker							
	PRX	S100	PMP22	NGFR	NES	NSE	MBP	CGA
Primary tumours	10/10	10/10	19/20	13/20	9/20	3/20	2/20	0/20
Metastases	10/10	10/10	9/10	8/10	4/10	1/10	0/9	0/9
Total positive	30/30	30/30	28/30	21/30	13/30	4/30	2/29	0/29
Labelling	Strong	Moderate - weak	Moderate - Strong	Weak - moderate	Weak	Weak - moderate	Weak	None

DFTD, devil facial tumour disease; PRX, periaxin; PMP22, peripheral myelin protein 22; NGFR, nerve growth factor receptor; NES, nestin; NSE, neuron specific enolase; MBP, myelin basic protein; CGA, chromogranin A. The table indicates the number of samples showing positive immunoreactivity for the marker in more than 10% of the viable-looking cells.

S100 are calcium-binding proteins commonly used as markers for neuronal cells, Schwann cells and tumours of neuronal origin. S100 expression was observed in all primary tumour samples (n = 20) and metastases (n = 10). Peripheral nerves also showed strong immunoreactivity for S100 (Figure 2.1.E-F).

PMP22 is an integral membrane protein that is a major component of myelin in the peripheral nervous system. Moderate PMP22 expression was detected in 95% of primary tumours (19/20) and 90% of metastases (9/10). Peripheral nerves bundles showed strong immunoreactivity for PMP22 (Figure 2.1.G-H).

NGFR, also called p75 (NTR), is expressed by both neuronal and glial cell types. NGFR expression in Schwann cells is important for the remyelination process after peripheral nerve injury (Tomita *et al.*, 2007). NGFR expression was observed in 65% of primary DFTD tumours (13/20) and 80% of metastases (8/10). Peripheral nerve bundles showed strong immunoreactivity for NGFR (Figure 2.1.I-J).

Nestin is an intermediate filament expressed during the development of the nervous system in both neuronal and glial cells. In DFTD cells, immunoreactivity for nestin was found in less than 50% of the primary tumour samples (9/20) and DFTD metastases (4/10). Devil's spinal cord tissue showed cells strongly labelled with nestin antibody (Figure 2.1.K-L).

Weak to moderate labelling for neuron specific enolase (NSE), a protein commonly found in neuronal and neuroendocrine cells, was only observed in 3/20 primary tumours and 1/10 metastasis. Immunoreactivity for NSE was detected in peripheral nerves (Figure 2.1.M-N) and spinal cord.

The protein encoded by the MBP gene is a major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system. MBP protein expression was only observed in 2/20 primary DFTD tumours. MBP was not detected in DFTD metastases. Although peripheral nerve bundles labelled positively with MBP, the interpretation of the results was difficult due the high background observed in the surrounding connective tissue (Figure 2.1.O-P).

Chromogranin A (CGA) belongs to a family of proteins found in the secretory granules of neuroendocrine cells. No immunoreactivity was found for CGA in DFTD tumour cells. However, strong granular and cytoplasmic immunoreactivity was observed in pituitary gland (Figure 2.1.Q-R) and cells of the islets of Langerhans in the pancreas.

2.3.3 Protein expression of myelin and neuronal markers in DFTD cell lines

A DFTD cell line was also evaluated for their expression of neuronal and peripheral nerve markers using flow cytometry. Cell surface or cytoplasmic expression was investigated in intact or permeabilized cells respectively. Cytoplasmic expression was clearly detected for periaxin, NGFR and nestin. Myelin protein zero (MPZ), which is a major integral membrane protein of the peripheral nervous system was also detected in permeabilized cells. Less conclusive cytoplasmic expression was observed with PMP22 and CGA. No surface expression was detected with any of the antibodies (Figure 2.2). NSE and MBP were not detected in permeabilized or non-permeabilized DFTD cells.

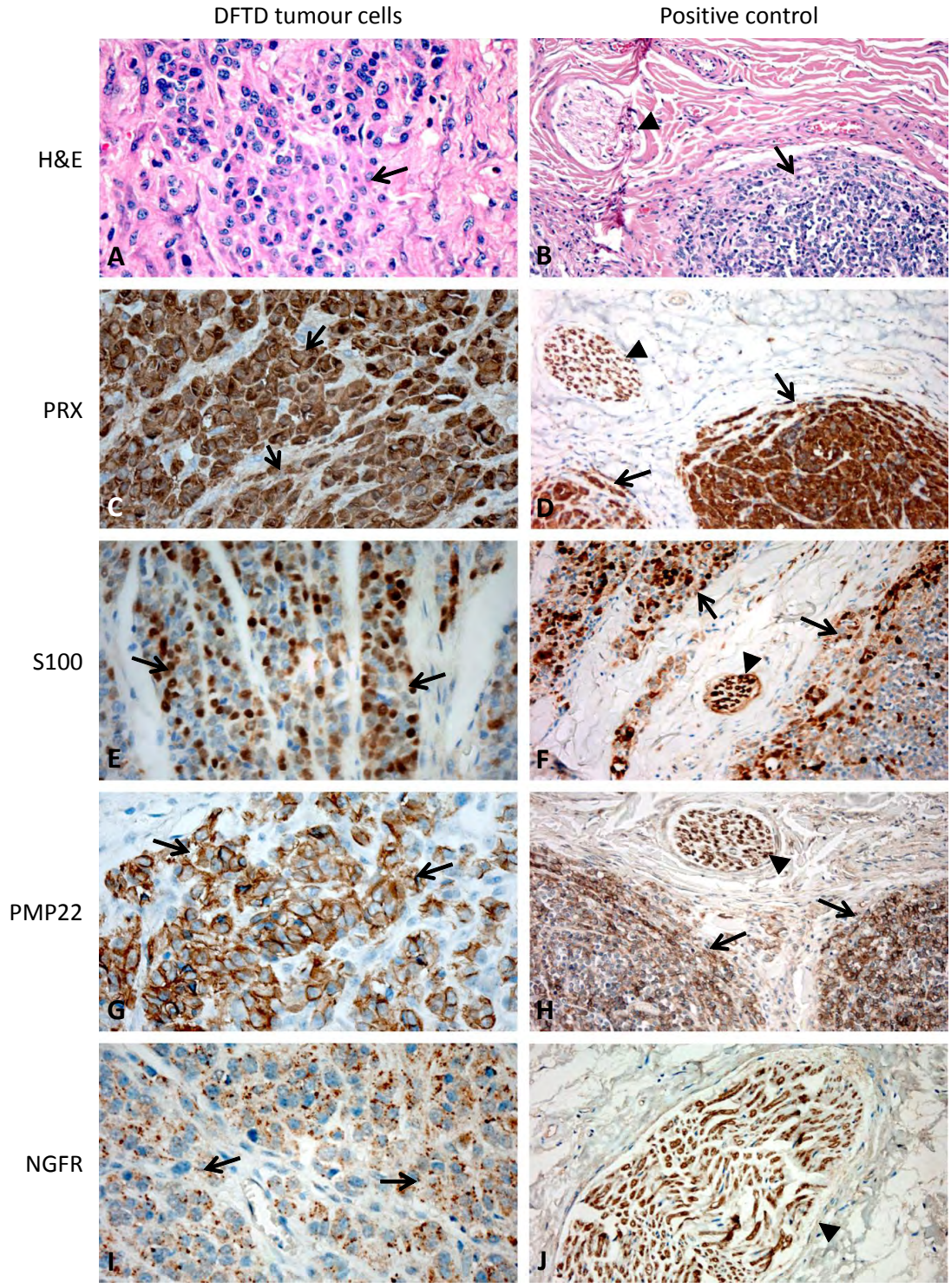

Figure 2.1 Expression of neuronal and peripheral nerve markers in DFTD and devil tissues. Left panels show labelling in primary DFTD tumour cells (arrows). Right panels show peripheral nerve tissue as a control (indicated with arrowheads) except for nestin (NES) and chromogranin A (CGA) in which positive control are spinal cord and pituitary gland respectively. **A-B**, DFTD tumours form dense aggregates arising in the dermis and propagate through the connective tissue. **C-D**. Strong and specific immunolabelling of DFTD tumour cells and peripheral nerve bundles with periaxin (PRX). **E-F**, Immunolabelling of DFTD tumour cells with S100 protein. Peripheral nerves (F) show strong S100 immunoreactivity. **G-H**, Strong peripheral myelin protein (PMP22) expression in DFTD tumour cells and peripheral nerves. **I-J**, Granular immunolabelling of DFTD tumour cells and peripheral nerve with nerve growth factor receptor (NGFR).

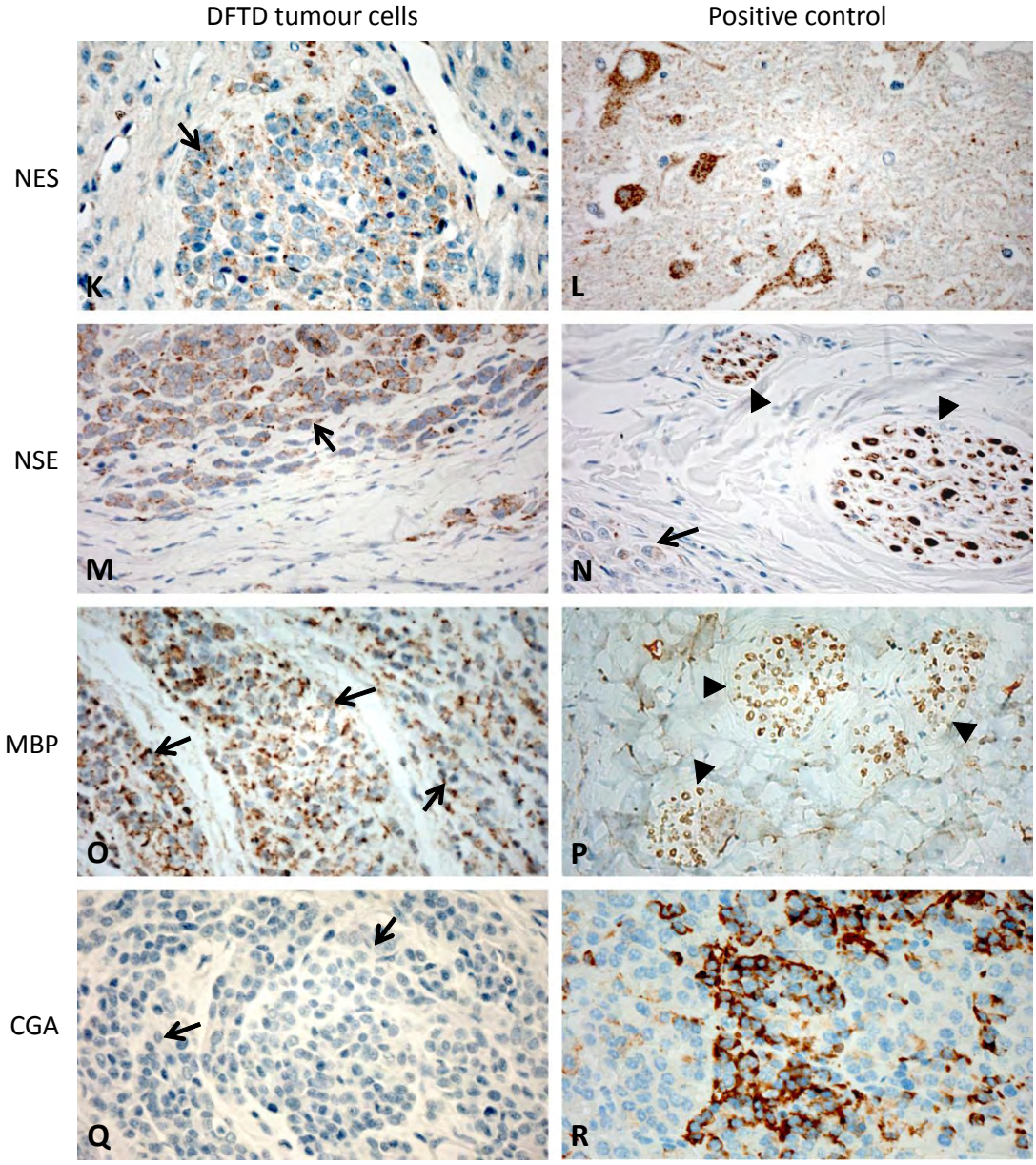

Figure 2.1(continued), K, Few cells within the tumour nest show nestin expression. **L**, Strong nestin labelling was detected in neuronal cells within the spinal cord. **M**, Immunoreactivity of neuron specific enolase (NSE) in DFTD tumour cells. **N**, Strong NSE labelling in peripheral nerves. **O**, Granular immunolabelling with myelin basic protein (MBP). High levels of non-specific labelling are observed in connective tissue. **P,** Moderate MBP labelling in peripheral nerves. **Q**, CGA immunoreactivity was not detected in any of the DFTD samples. **R,** CGA expression was observed in cells of the devil's anterior pituitary gland. A-B, hematoxylin and eosin staining. C-R immunohistochemical detection with EnVision+ system (Dako) with hematoxylin counterstain.

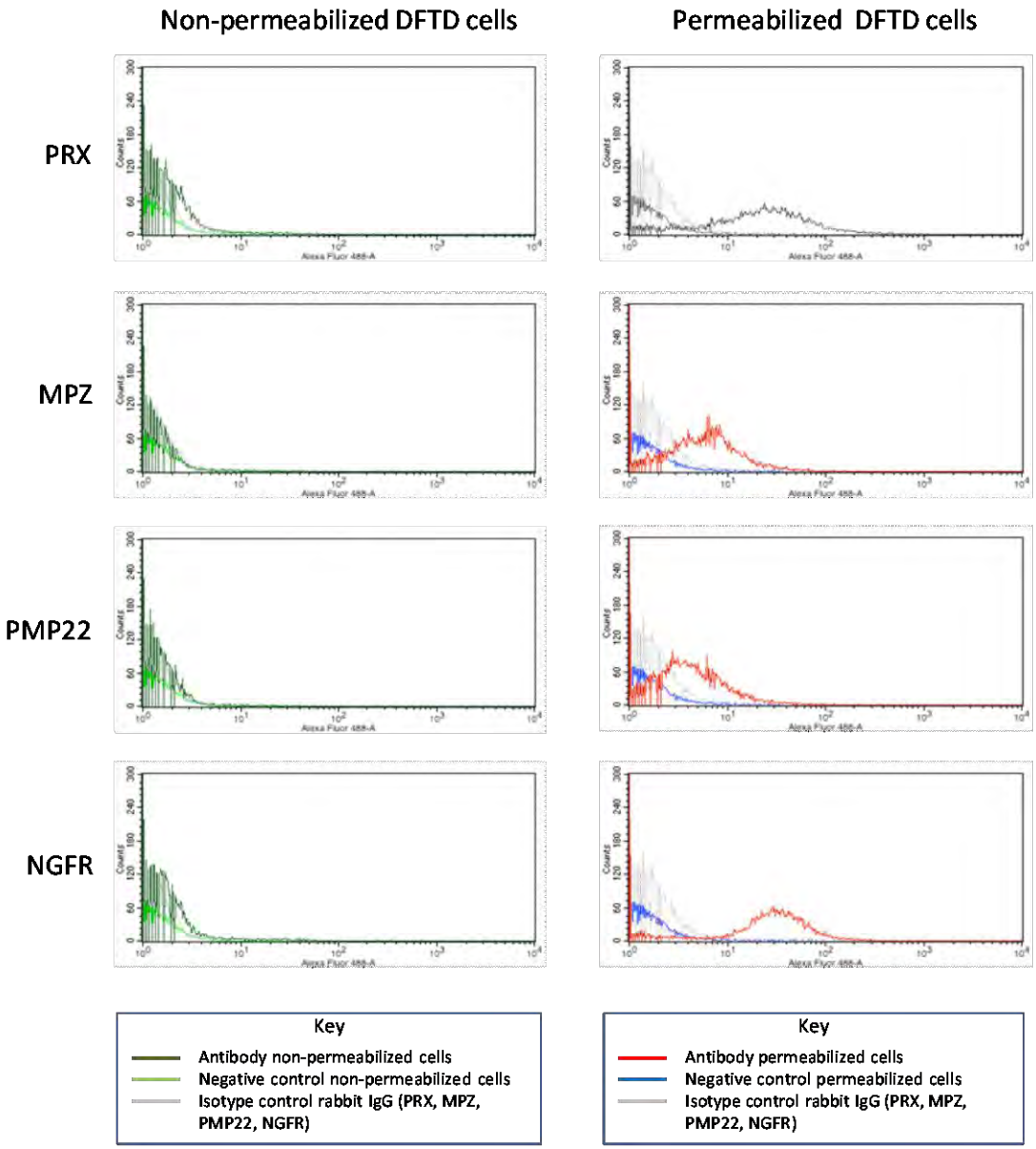

Figure 2.2 Expression of peripheral nerve and myelin markers in DFTD cell lines. Flow cytometry histograms representing surface expression (labelling in non-permeabilized cells) or cytoplasmic expression (permeabilized cells). Positive immunolabelling is represented by a shift to the right in fluorescence intensity (X-axis) compared to that of the negative or isotype control. No cell surface expression was detected with any of the antibodies. Cytoplasmic expression was detected in DFTD cells labelled with periaxin (PRX), myelin protein zero (MPZ) and nerve growth factor receptor (NGFR). Less conclusive evidence of cytoplasmic expression was observed for peripheral myelin protein 22 (PMP22).

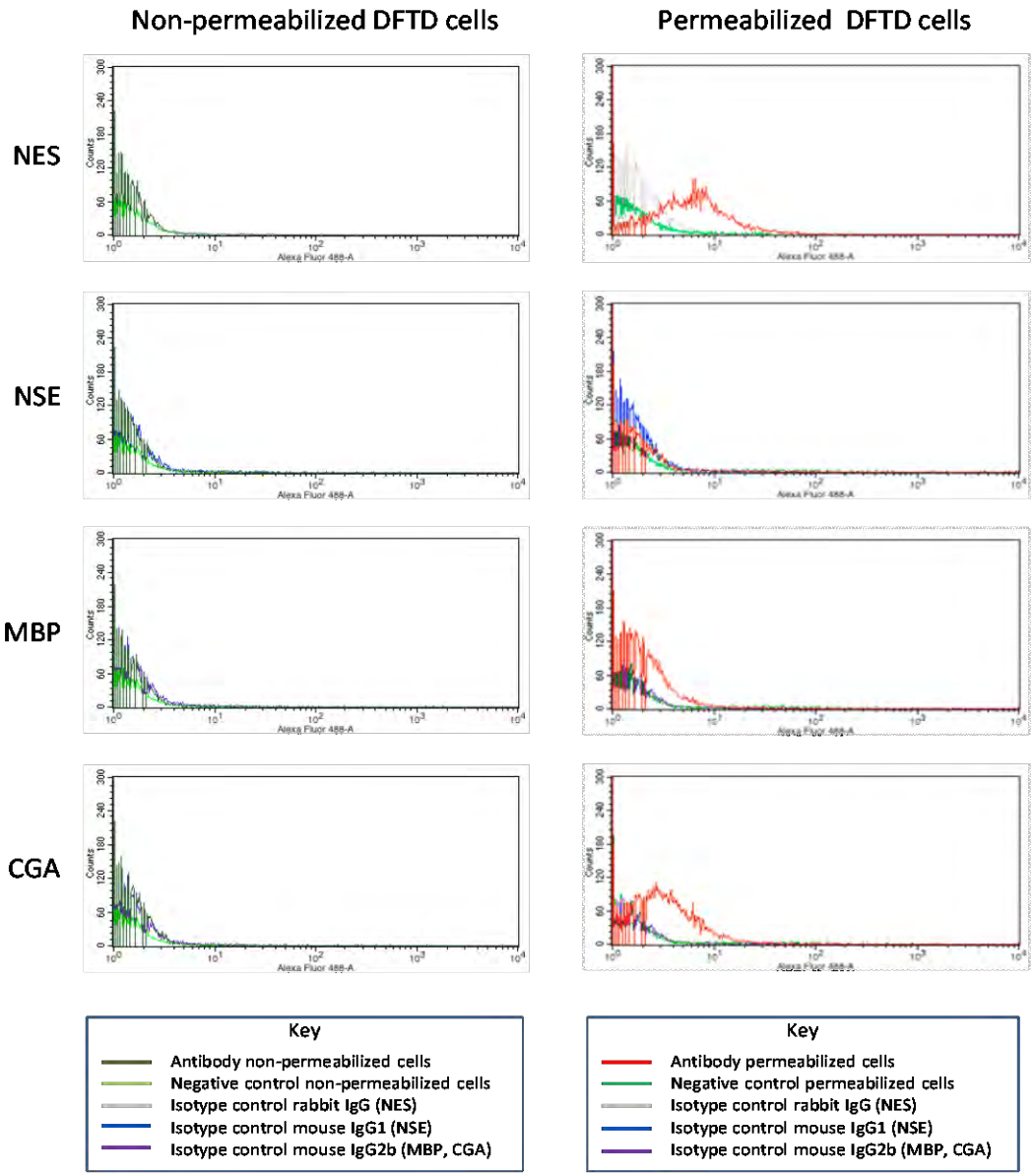

Figure 2.2 (Continued), cytoplasmic expression was detected for nestin (NES). Less conclusive evidence of cytoplasmic expression was detected for chromogranin A (CGA). Neuron specific enolase (NSE) and myelin basic protein (MBP) were not detected in the surface or in the cytoplasm.

2.4 Discussion

Recent studies on the Tasmanian devil transcriptome revealed that devil facial tumour cells express a set of genes related to the myelination pathway in the peripheral nervous system (PNS) (Murchison *et al.*, 2010). The immunohistochemistry and flow cytometry studies confirmed the protein expression of a number of markers found throughout the neuronal system in DFTD. These included the neuronal markers protein S100, neuronal specific enolase (NSE), nestin (NES) and myelin basic protein (MBP). Notably, DFTD tumour cells expressed structural proteins exclusively found in myelinating Schwann cells in the peripheral nervous system. These included peripheral myelin protein 22 (MP22), myelin protein zero (MPZ), and the recently described periaxin (PRX). Nerve growth factor receptor (NGFR), which is involved in the differentiation of Schwann cells, was also detected in DFTD tumour cells. Based on these combined results, this thesis proposes that DFTD is a peripheral nerve sheath tumour of Schwann cell origin.

The first immunohistological study of DFTD revealed labelling characteristics consistent with cells of neuroendocrine origin (Loh *et al.*, 2006b). The neural crest, which arises from the neuroectoderm, gives rise to a number of cell types during vertebrate embryogenesis. These neural crest derived cells include neurons and glial cells from the peripheral nervous system, pigment cells in the skin (melanocytes); endocrine cells, and a variety of mesenchymal cell types (Dupin *et al.*, 2007, Joseph *et al.*, 2004, Le Douarin *et al.*, 2008). This thesis provided additional support to the neuroectodermal origin of DFTD by detecting immunoreactivity to nestin and NGFR, proteins found in neuronal cells and neuronal neoplasias (Hoshi *et al.*, 1994, Shimada *et al.*, 2007). However, chromogranin A (CGA), a glycoprotein that is found in secretory vesicles of neurons and endocrine cells (Hendy *et al.*, 1995), was not detected in DFTD tumours. The presence of Schwann cell markers in DFTD and the lack of CGA expression indicate that DFTD tumours originated from cells of the peripheral nervous system lineage rather than neuroendocrine as originally proposed.

Differences with the previous study can be attributed to improvements in the technique used. Applying immunohistochemistry to the study of protein expression in marsupials tissues have been challenging (Canfield and Hemsley, 2000, Loh *et al.*, 2006b). One of the major constraints is the limited availability of commercial antibodies targeted to

marsupial tissues. This is especially relevant for antibodies against antigens that are not widely studied, such as myelin proteins. In this current study of devil tissue, it was found that some antibodies, in particular polyclonal antibodies, could produce relatively high levels of non-specific labelling in connective and other tissues. Moreover, secondary avidin or biotin conjugated antibodies produced high background and interpretation of results was difficult. This non-specific labelling was notably reduced with a biotin-free visualisation system and by selecting monoclonal antibodies.

The major difference to the original immunohistochemical characterization of DFTD was the detection of CGA. The Loh *et al.* study detected CGA in all the samples and was described as "low intensity positivity in the cytoplasm". In this thesis, a monoclonal anti-CGA was used rather than a polyclonal antibody and detection was achieved using the EnVision+ system (Dako) which is an avidin-biotin free system. With this method, strong CGA expression was observed in samples of pituitary gland and pancreas. However, none of the DFTD samples showed CGA expression. The polyclonal anti-CGA antibody used in Loh *et al.* study was re-tested in eight tumour samples used in this chapter and devil tissues using the avidin-biotin free system. Although CGA was detected in pituitary gland and pancreas, none of the eight samples showed CGA expression (Appendix 3). The first study used tissue samples collected from 2001 to 2004. It could be speculated that evolution of the tumour could account for the difference as the earliest samples used in the current research were from 2006. Testing this hypothesis would have required analysing early tumour samples; however, the samples were not available for the current study.

The flow cytometry analysis clearly detected periaxin, NGFR, nestin and MPZ within the cytoplasm of DFTD cells in culture, but not the other markers. None of the markers was detected on the cell surface. However, as there are not available appropriate cultured devil cells that can be used as positive controls, precaution needs to be taken for the interpretation of these results. It is possible that the cells in culture do not express the markers but is also possible that the antibodies are simply not suitable for use in flow cytometry. The current study was able to establish a primary culture of devil' Schwann cells and fibroblasts suitable for immunofluorescence studies (see Chapter 4). However, flow cytometry requires a large number of cells that remain viable in cell suspension.

Refinement of the cell culture is an ongoing process and this could make available control devil cells for use in flow cytometry in the future.

Cytoplasmic expression (in permeabilized cells) was clearly detected for periaxin, NGFR and nestin. Myelin protein zero (MPZ), which is a major integral membrane protein of the peripheral nervous system was also detected in permeabilized cells. Less conclusive cytoplasmic expression was observed with PMP22 and CGA. No surface expression was detected with any of the antibodies (Figure 2.2). NSE and MBP were not detected in permeabilized or non-permeabilized DFTD cells. However, there are not available appropriate devil cells that can be used as positive control for these two markers. Therefore is still possible that the antibodies are simply not suitable for use in flow cytometry.

Tumours of the PNS have been described in humans, dogs, cattle and other animals (Bundza *et al.*, 1986, Chijiwa *et al.*, 2004, Nielsen *et al.*, 2007, Schoniger and Summers, 2009, Schulman *et al.*, 2009). Furthermore, the Schwann cell is thought to be the major contributor to the formation of both benign and malignant peripheral nerve sheath tumours neoplasms (Carroll and Ratner, 2008, Gupta *et al.*, 2008, Zhu *et al.*, 2002). Tumours of the PNS can occur sporadically but the majority arise in individuals affected with neurofibromatosis type 1 (NF1) and type 2 (NF2) (Ferner, 2010, Stemmer-Rachamimov *et al.*, 2004). NF1 and NF2 are autosomal dominant tumour-suppressor gene syndromes and evidence provided by mouse models indicates that loss of NF1 or NF2 functionality in the Schwann cell lineage is sufficient to generate tumours (Carroll and Ratner, 2008, Le *et al.*, 2011, Zheng *et al.*, 2008, Zhu *et al.*, 2002). The expression of these cancer genes in DFTD requires further investigation.

Evidence from human cancers indicates that tumours have a phenotypic and functional heterogeneous composition of cells. It has also been noted that the cell that acquires the first cancer-promoting mutations (cell-of-origin) is not necessarily related to the cancer stem cell (CSC), the cellular subset within the tumour that sustains malignant growth (Visvader, 2011). In this context, it is remarkable that DFTD tumours consistently express markers of highly differentiated Schwann cells. As DFTD propagates as a clone, it is likely that these differentiated cells reflect both, the cell of origin and the cancer propagating cells. A similar mechanism has been described in mouse models of

neurofibromas and peripheral malignant nerve sheath tumours (MPNST), where tumours developed from fully differentiated glial cells in adult sciatic nerves (Zheng *et al.*, 2008).

Biting has been proposed as the most likely mean of DFTD transmission (Obendorf and McGlashan, 2008). It could be speculated that the origin of DFTD also relates to the characteristic biting behaviour of Tasmanian devils during mating and feeding. Frequent injury and infection are common in the face in areas highly innervated such as the devils' vibrissae. Chronic inflammation, a common feature of infections, has been associated with cancer due to the generation of free radicals, prostaglandins, cytokines and other mediators that participate in the inflammatory response. Prolonged exposure to these mediators leads to increased cell proliferation, mutagenesis, oncogene activation, and angiogenesis (Shacter and Weitzman, 2002). These factors could particularly affect tissue stem cells that participate in tissue regeneration and it is increasingly accepted that carcinogenesis develops by misappropriating homeostatic mechanisms that govern tissue repair and stem cell-renewal (Beachy *et al.*, 2004). Mature Schwann cells are able to dedifferentiate and actively participate in nerve repair after injury (Dahlin, 2008). Schwann cells also modulate local immune reactions in the peripheral nervous system (Gold *et al.*, 1999). Interestingly, nestin a marker of stem cells (Lendahl *et al.*, 1990) was also detected in DFTD tumour cells. Therefore, the plasticity and immunocompetence of Schwann cells may be significant in the origin and evolution of DFTD as a transmissible cancer.

In summary, the results of this chapter confirmed the protein expression of genes involved in the myelination pathway within the peripheral nervous system. Thus, the combined data of the previous genetic studies and the protein profile presented here indicate that DFTD tumour cells are from the peripheral nervous system lineage and most likely to be of Schwann cell origin.

A correct classification of the cells of origin of tumours is important for the biological understanding of carcinogenesis and to tailor new approaches for the prevention and treatment of tumours (Waldum *et al.*, 2008). The finding that DFTD originated from Schwann cells in the peripheral nervous system clearly raises new opportunities for the investigation of diagnostic markers for the disease and the understanding of the immune evasion of this transmissible cancer.

3 Diagnostic markers for devil facial tumour disease

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3.1 Introduction

Diagnosis of devil facial tumour disease (DFTD) is currently based on histopathology, clinical appearance of the disease in affected animals and cytogenetics. Karyotyping is the most accurate method for tumour diagnosis. This classical analysis requires chromosomal banding and collection of fresh tumour biopsies for establishment of cell cultures in vitro. This is not only time-consuming but also labour intensive. As Tasmanian devils are also susceptible to other types of neoplasias (Canfield and Cunningham, 1993, Griner, 1979) , a specific diagnostic test that can be carried out rapidly on fixed samples is required to differentiate DFTD tumours from other cancers of similar morphological appearance (Jones *et al.*, 2007).

The previous chapter showed that DFTD tumours consistently express a set of proteins related to the myelination pathway in the peripheral nervous system (PNS). Based on the results it was proposed that DFTD is of Schwann cell origin. The aim of this chapter was to evaluate the utility of the neuronal and peripheral nerve proteins as diagnostic markers of DFTD. To achieve this, a thorough semi-quantitative assessment of the pattern of immunohistochemical expression of these markers was performed on DFTD and non-DFTD tumours, and Tasmanian devil tissues.

Additionally, the utility of the markers was evaluated in alternative models of the disease such as murine xenografted DFTD tumours, DFTD cell lines and devil Schwann cells in primary culture. Results of this chapter have been published in Tovar *et al.,* (2011) and Kreiss *et al.,* (2011b).

3.2 Methods

3.2.1 Immunohistochemistry

Devil tissues, DFTD tumours and non-DFTD tumour samples were supplied by the tissue bank held at the Menzies Research Institute Tasmania, University of Tasmania, and the Mount Pleasant Laboratories from the Tasmanian Department of Primary Industry, Parks, Water and Environment (DPIPWE). Samples from primary DFTD tumours, DFTD metastases and normal devil tissues were evaluated by immunohistochemistry for the expression of periaxin, S100 protein, peripheral myelin protein 22, nerve growth factor receptor, nestin, neuron specific enolase, myelin basic protein and chromogranin A. Tissue sections preparation and immunohistochemistry protocols were as described in Chapter 2.2.

3.2.2 Annotation and interpretation of protein expression in tissues

A light microscope (Olympus-BX50) coupled with a camera (Leica-DFC320) was used for visualization and acquisition of the images. Labelling reactions, analyzed semi-quantitatively by the principal author and a trained veterinary pathologist, were assessed according to the following criteria: *i*) immuno-labelling intensity (negative, weak, moderate or strong) compared with that of the positive control; positive labelling was defined by the presence of 10% or more immunoreactive cells in tumour sections; *ii*) the fraction of tumour cells labelled was defined as 10-25%, 25-50%, 50-75%, or >75%; and *iii*) sub-cellular localization (nucleus, cytoplasmic or associated with the cell membrane) combined with parameters describing the labelling characteristics (i.e. smooth, granular, or fine granular).

3.2.3 Primary Schwann cell culture

The following protocol was modified from Mauritz *et al*., (2004). Sciatic nerves were collected into Dulbecco's modified Eagle medium (DMEM; Gibco, Auckland, New Zealand) during autopsy of an adult male devil, and the epineurium was dissected with micro-scissors and forceps. The epineurium-free tissue was incubated for 14 days at 37 °C and 5% CO_2 in DMEM containing 10% fetal calf serum (FCS; Gibco), 1% penicillin/streptomycin and 2 µM forskolin (Sigma, St Louis, MO). Nerve fascicles were washed gently with DMEM and then incubated with DMEM containing 10% FCS, 50 U of penicillin, 50 µg of streptomycin (penicillin-streptomycin liquid, Sigma) and an enzyme mixture consisting of 0.15 U/ml collagenase and 0.24 U/ml dispase (Invitrogen, Carlsbad, CA) at 37 °C and 5% CO_2 for 20 hours. After dissociation through glass pipettes, the

homogenous cell suspension was centrifuged at 250 × *g* for five minutes at 21 °C. Following resuspension, the cells were filtered through 70-µm cell strainer to remove undigested tissue. The cell filtrate was plated into a tissue culture flask and incubated at 37 °C and 5% CO_2 in DMEM culture media containing 2 µM forskolin, 10 ng/ml fibroblast growth factor (Promega, Madison, WI), 5 µg/ml bovine pituitary extract (Sigma) and 1% penicillin-streptomycin liquid (Invitrogen). Two weeks later the cells were harvested for immunofluorescence analysis.

3.2.4 Immunofluorescence

Cultured DFTD tumour cells and primary cultured devil Schwann cells were plated onto Poly-L-Lysine (Sigma)-coated coverslips and incubated to semi-confluence at 37 °C and 5% CO_2. DFTD tumour cells were incubated in complete RPMI medium (RPMI 1640, Gibco) containing 10% FCS, 2 mM L-glutamine (Sigma) and 40 mg/ml gentamicin (Pfizer, Sydney, Australia). Schwann cells were incubated in the DMEM culture medium described above. The cells were fixed in 4% paraformaldehyde-glucose (Sigma) for 30 minutes followed by three washes with PBS. Cells were then incubated in 10% BSA for an hour to block non-specific binding. Primary antibody was incubated in 0.3% Triton X100 overnight at 4 °C. After washing with PBS the cells were incubated in the dark with a fluorescently labeled secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG; Invitrogen) in PBS for an hour at room temperature. Actin stain (Alexa Fluor 488 phalloidin; Invitrogen) was added and incubated for 30 minutes. The coverslips were washed with PBS and rinsed with distilled water prior to mounting onto glass slides using fluorescent mounting medium (Dako). Labelling was visualized under a confocal microscope (Zeiss LSM 510 Meta). Isotype and negative (omitting the primary antibodies) controls were also performed in parallel.

3.2.5 Murine xenografted DFTD tumours

A DFTD xenograft model in immuno-compromised NOD/SCID mice was already described (Kreiss *et al.*, 2011b) In brief, NOD/SCID mice were injected subcutaneously with viable DFTD tumour cells. All inoculated mice developed tumours. Additionally, cells derived from these tumours were successfully passaged into other NOD/SCID mice.

3.3 Results

3.3.1 Patterns of expression of Schwann cell markers in DFTD and devil tissues

The previous chapter demonstrated that DFTD expresses a number of Schwann cell markers. These include periaxin (PRX), S100 protein, peripheral myelin protein 22 (PMP22), nerve growth factor receptor (NGFR), nestin (NES), neuron specific enolase (NSE) and myelin basic protein (MBP). This chapter presents a thorough examination of the immunohistochemical labelling patterns of these markers in DFTD. The semi-quantitative evaluation of the labelling included the fraction of labelled cells, cellular localization and description of the characteristics of the stain (Table 3.1). This chapter also presents the pattern of expression of the markers in normal devil tissues (Table 3.2). The results for positive control were already reported in the previous chapter and therefore they are not included here.

Of the eight markers assessed, periaxin, a marker of myelinating Schwann cells (Gillespie *et al.*, 1994), gave the most consistent labelling of tumour cells. In all cases, the labelling was moderate to strong, cytoplasmic and observed in more than 75% of the tumour cells. The periaxin expression was observed in all DFTD primary tumours and metastases. Although 75% was used as the criteria for reporting the fraction of labelled cells, it was observed that periaxin consistently labelled almost 100% of the tumour cells in all the samples. Stronger immunoreactivity was occasionally observed at the edges of the tumour fascicles. One particular feature of periaxin was its strong specificity for DFTD tumour cells and the lack of background labelling in other tissues (Figure 3.1.A). Peripheral nerves within these DFTD tumour sections were also clearly identified by their strong and specific immunoreactivity for periaxin.

S100 protein is a commonly used marker for peripheral nerve sheath tumours (Coindre, 2003). S100 was observed in all DFTD samples. However, the intensity of the labelling was variable between samples and within a sample; background labelling was also observed in other tissues (Tables 3.1 and 3.2). In DFTD tumour cells, labelling with S100 was consistently granular and located in both cytoplasmic and nuclear compartments (Figure 3.1.B). Similar to periaxin, the level of S100 expression varied across tumour nodules (i.e. the cells at the periphery of the tumour exhibited stronger immunoreactivity compared to cells in the centre). Strong S100 immunoreactivity was also observed in peripheral nerves and in the nucleus of neurons and oligodendrocytes located in devil spinal cord.

Table 3.1 Patterns of expression of myelin and neuronal markers in DFTD primary tumours and metastases

	PRX		S100		PMP22		NGFR		NES		NSE		MBP		CGA	
	PT	M	PT	M	PT	M	PT	M	PT	M	PT	M	PT	M	PT	M
Intensity																
Strong:	15/20	7/10	4/20	1/10	5/20	0/10	1/20	0/10	0/20	0/10	0/20	0/10	0/20	0/9	0/20	0/9
Moderate:	5/20	2/10	8/20	6/10	6/20	1/10	5/20	1/10	2/20	1/10	1/20	0/10	0/20	0/9	0/20	0/9
Weak:	0/20	1/10	8/20	3/10	8/20	8/10	7/20	7/10	7/20	3/10	2/20	1/10	2/20	0/9	0/20	0/9
Negative:	0/20	0/10	0/20	0/10	1/20	1/10	7/20	2/10	11/20	6/10	17/20	9/10	18/20	0/9	20/20	9/9
Total positive:	20/20	10/10	20/20	10/10	19/20	9/10	13/20	8/10	9/20	4/10	3/20	1/10	2/20	0/9	0/20	0/9
% positive:	100%	100%	100%	100%	95%	90%	65%	80%	45%	40%	15%	10%	10%	0%	0%	0%
Fraction of positive cells	20/20	10/10	1/20	1/10	11/19	3/9	1/13	2/8	1/9	0/4	0/3	0/1	1/2	0/0	0/0	0/0
>75%:	0/20	0/10	5/20	1/10	3/19	4/9	7/13	4/8	2/9	2/4	0/3	0/1	1/2	0/0	0/0	0/0
75-50%:	0/20	0/10	7/20	5/10	4/19	1/9	2/13	1/8	2/9	0/4	1/3	0/1	0/2	0/0	0/0	0/0
50-25%:	0/20	0/10	7/20	3/10	1/19	1/9	3/13	1/8	4/9	2/4	2/3	1/1	0/2	0/0	0/0	0/0
25-10%																
Labelling																
Smooth:	20/20	10/10	4/20	3/10	8/19	4/9	0/13	0/8	0/9	0/4	0/3	0/1	0/2	0/0	0/0	0/0
Fine granular:	0/20	0/10	0/20	0/10	2/19	4/9	0/13	0/8	2/9	1/4	1/3	0/1	0/2	0/0	0/0	0/0
Granular:	0/20	0/10	16/20	7/10	9/19	1/9	13/13	8/8	7/9	3/4	2/3	1/1	2/2	0/0	0/0	0/0
Localization																
Memb. association:	2/20	0/10	0/20	0/10	11/19	4/9	0/13	0/8	0/9	0/4	0/3	0/1	0/2	0/0	0/0	0/0
Cytoplasm:	18/20	8/10	7/20	1/10	8/19	5/9	13/13	8/8	9/9	4/4	3/3	1/1	2/2	0/0	0/0	0/0
Cyto.-nucleus:	0/20	2/10	13/20	9/10	0/19	0/9	0/13	0/8	0/9	0/4	0/3	0/1	0/2	0/0	0/0	0/0

DFTD, devil facial tumour disease; PRX, periaxin; S100, S100 protein; PMP22, peripheral myelin protein 22; NGFR, nerve growth factor receptor; NES, nestin; NSE, neuron specific enolase; MBP, myelin basic protein; CGA, chromogranin A; PT: primary tumours; M: metastases.

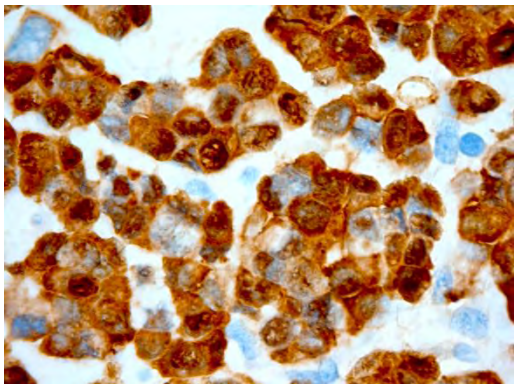
Table 3.2 Pattern of expression of selected markers in normal devil tissues.

	PRX	S100	PMP22	NGFR	NES	NSE	MBP	CGA
Epithelium	-	-	-	-	-	-	++	-
Connective tissue	-	+	++	+	-	+	+++	-
Sebaceous glands	-	-	-	-	+	-	-	+
Hair follicles	-	+	-	-	-	+	-	+
Endothelium	-	+	-	-	-	-	-	-
Skeletal muscle	-	++	-	-	+++	-	-	+
Peripheral nerves	+++	+++	+++	+++	-	+++	++	-
Anterior pituitary gland	-	+	++	++	++	+	+	+++
Posterior pituitary gland	-	+++	+	+	-	++	+	-
Lymphoid tissue – leukocytes	-	-	+	+	-	-	-	-
Spinal Cord	-	++	+	+++	+++	++	+	-
Kidney	+	++	-	+	-	-	-	-
Pancreas	ND	ND	ND	ND	ND	ND	ND	+++

PRX, periaxin; PMP22, peripheral myelin protein 22; S100, S100 protein; NGFR, nerve growth factor receptor; NES, nestin; NSE, neuron specific enolase; MBP, myelin basic protein; CGA, chromogranin A; ND not done; - negative; + low intensity; ++ moderate; +++ high intensity.

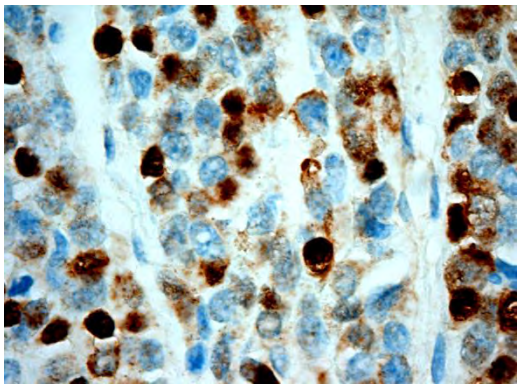
A. Periaxin (PRX)
Overall positivity: 30/30 (100%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	15/20	7/10	>75%:	20/20	10/10
Moderate:	5/20	2/10	75-50%:	0/20	0/10
Weak:	0/20	1/10	50-25%:	0/20	0/10
Negative:	0/20	0/10	25-10%	0/20	0/10
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	20/20	10/10	Memb. assoc:	2/20	0/10
Fine gran.:	0/20	0/10	Cytoplasm:	18/20	8/10
Granular:	0/20	0/10	Cyto.-Nucleus:	0/20	2/10



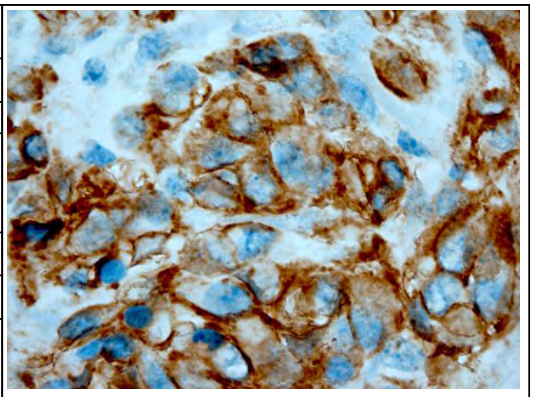
B. Protein S100 (S100)
Overall positivity: 30/30 (100%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	4/20	0/10	>75%:	1/20	1/10
Moderate:	8/20	6/10	75-50%:	5/20	1/10
Weak:	8/20	3/10	50-25%:	7/20	5/10
Negative:	0/20	1/10	25-10%:	7/20	3/10
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	4/20	3/10	Memb. assoc:	0/20	0/10
Fine gran.:	0/20	0/10	Cytoplasm:	7/20	1/10
Granular	16/20	7/10	Cyto.-Nucleus:	13/20	9/10



C. Peripheral Myelin Protein 22 (PMP22)
Overall positivity: 28/30 (93%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	5/20	0/10	>75%:	11/19	3/9
Moderate:	6/20	1/10	75-50%:	3/19	4/9
Weak:	8/20	8/10	50-25%:	4/19	1/9
Negative:	1/20	1/10	25-10%:	1/19	1/9
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	8/19	4/9	Memb. assoc:	11/19	4/9
Fine gran.:	2/19	4/9	Cytoplasm:	8/19	5/9
Granular:	9/19	1/9	Nucleus:	0/19	0/9



D. Nerve Growth Factor Receptor (NGFR)
Overall positivity: 21/30 (70%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	1/20	0/10	>75%:	1/13	2/8
Moderate:	5/20	1/10	75-50%:	7/13	4/8
Weak:	7/20	7/10	50-25%:	2/13	1/8
Negative:	7/20	2/10	25-10%:	3/13	1/8
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	0/13	0/8	Memb. assoc:	0/13	0/8
Fine gran.:	0/13	0/8	Cytoplasm:	13/13	8/8
Granular	13/13	8/8	Nucleus:	0/13	0/8

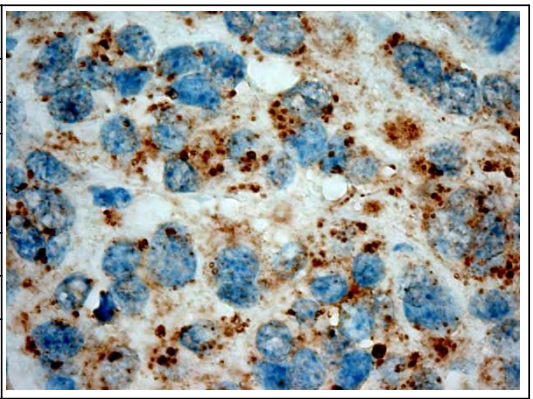
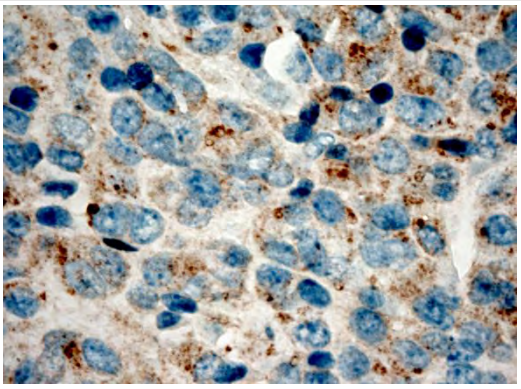


Figure 3.1 Pattern of expression of neuronal and myelin markers in DFTD tumour cells. Immunoreactivity was assessed semiquantitatively including intensity, fraction of positive cells, and localization and characteristic of the labelling.

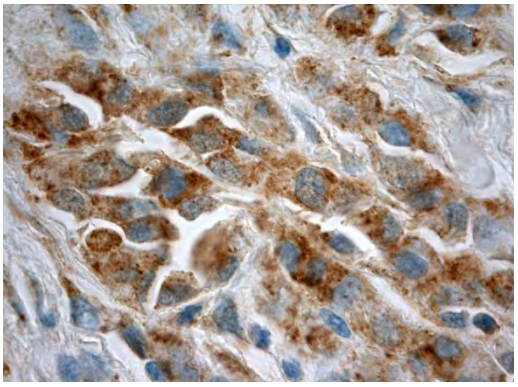
E. Nestin (NES)
Overall positivity: 13/30 (43%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	0/20	0/10	>75%:	1/9	0/4
Moderate:	2/20	1/10	75-50%:	2/9	2/4
Weak:	7/20	3/10	50-25%:	2/9	0/4
Negative:	11/20	6/10	25-10%	4/9	2/4
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	0/9	0/4	Memb. assoc:	0/9	0/4
Fine gran.:	2/9	1/4	Cytoplasm:	9/9	4/4
Granular:	7/9	3/4	Nucleus:	0/9	0/4



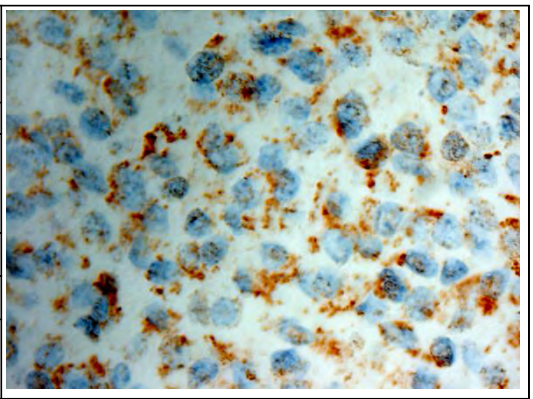
F. Neuron Specific Enolase (NSE)
Overall positivity: 4/30 (13%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	0/20	0/10	>75%:	0/3	0/1
Moderate:	1/20	0/10	75-50%:	0/3	0/1
Weak:	2/20	1/10	50-25%:	1/3	0/1
Negative:	17/20	9/10	25-10%:	2/3	1/1
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	0/3	0/1	Memb. assoc:	0/3	0/1
Fine gran.:	1/3	0/1	Cytoplasm:	3/3	1/1
Granular:	2/3	1/1	Nucleus:	0/3	0/1



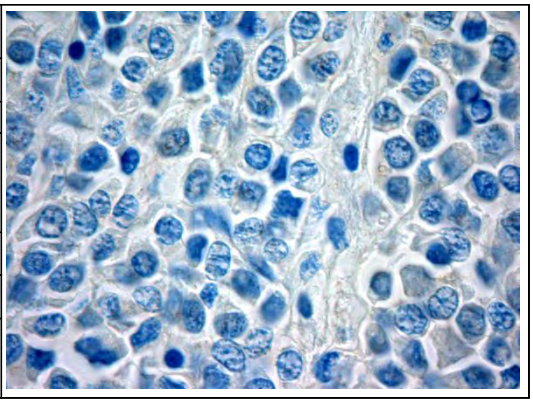
G. Myelin Basic Protein (MBP)
Overall positivity: 2/29 (7%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	0/20	0/9	>75%:	1/2	0/0
Moderate:	0/20	0/9	75-50%:	1/2	0/0
Weak:	2/20	0/9	50-25%:	0/2	0/0
Neg:	18/20	0/9	25-10%:	0/2	0/0
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	0/2	0/0	Memb. assoc:	0/2	0/0
Fine gran.:	0/2	0/0	Cytoplasm:	2/2	0/0
Granular:	2/2	0/0	Nucleus:	0/2	0/0



H. Chromogranin A (CGA)
Overall positivity: 0/29 (0%)

Intensity	PT	M	Fraction of positive cells	PT	M
Strong:	0/20	0/9	>75%:	0/0	0/0
Moderate:	0/20	0/9	75-50%:	0/0	0/0
Weak:	0/20	0/9	50-25%:	0/0	0/0
Neg:	20/20	9/9	25-10%:	0/0	0/0
Labelling			**Localization**		
	PT	M		PT	M
Smooth:	0/0	0/0	Memb. assoc:	0/0	0/0
Fine gran.:	0/0	0/0	Cytoplasm:	0/0	0/0
Granular:	0/0	0/0	Nucleus:	0/0	0/0



Figures 3.1 (continued) Right panels show immunoreactivity for each marker in DFTD tumour cells at 100× magnification. PT, primary tumour; M, metastases.

Strong immunoreactivity for PMP22, a major component of myelin in the peripheral nervous system, was observed at the outer margins of the tumour nodules and in neoplastic cells at the advancing edge of the tumour. PMP22 expression was smooth to fine granular. The labelling was predominantly cytoplasmic and frequently enhanced at the cell membrane (Figure 3.1.C). Although most of the samples were positive, labelling within a single sample, as indicated by the fraction of labelled cells, was highly variable ((table 3.1). Peripheral nerve bundles were also positive for PMP22. Intense non-specific background of the connective tissue was also frequently observed.

The labelling pattern for the nerve growth factor receptor (NGFR) was cytoplasmic and granular and it was observed in more than 50% of the DFTD tumour cells (Figure 3.1.D). Some populations of cells, usually located to the edges of the tumour fascicles, were strongly positive for NGFR. Immunoreactivity to NGFR was also observed in peripheral nerves, spinal cord and anterior and posterior pituitary gland. Occasional non-specific labelling was observed in connective tissues and in renal tubular epithelium (Table 3.2).

The labelling expression of nestin was weak to moderate and variable. Positive cells showed granular cytoplasmic labelling. A particular feature of nestin labelling (not observed with the other markers) was its tendency to form coarse granules or clusters within the cytoplasm of the cell (Figure 3.1.E). Skeletal muscle cells were also strongly positive for nestin whereas sebaceous glands showed weak immunoreactivity (Table 3.2).

Weak to moderate granular and cytoplasmic labelling for neuron specific enolase (NSE) was observed in 13% of samples (4/30) with less than 50% of cells in each sample being positive (Figure 3.1.F). Notably, cells at the periphery of the tumour nests showed stronger immunoreactivity. Immunoreactivity for NSE was also detected in peripheral nerves, spinal cord and pituitary gland. Weak background labelling was also observed in connective tissues and hair follicles.

Positivity for myelin basic protein (MBP) was only observed in 2/20 DFTD primary tumours. In these two samples more than 50% of the cells showed immunoreactivity. The labelling pattern was diffuse, granular and cytoplasmic (Figure 3.1.G). MBP expression was also observed in peripheral nerves. Significant patchy background labelling was present in many samples making overall interpretation difficult. For instance, non-specific

and strong labelling was observed in connective tissues, epidermis and dermis. Necrotic foci within the tumour also showed non-specific immunoreactivity.

No immunoreactivity was detected for chromogranin A (CGA) in DFTD viable tumour cells (Figure 3.1.H). However, strong granular and cytoplasmic immunoreactivity for this protein, which is normally found in endocrine cells (Hendy *et al.*, 1995), was observed in pituitary gland and cells of the islets of Langerhans in the pancreas. Background labelling was observed in hair follicles and muscle cells.

3.3.2 Periaxin expression in xenografted tumours, non-DFTD tumours and cultured Schwann cells

The results above showed that periaxin consistently gave the strongest and most specific labelling of DFTD tumour cells. Periaxin immunoreactivity was also detected in almost 100% of the tumour cells in both primary tumour and metastases (Table 3.1. Figures 3.2.A-D). These results suggested that periaxin could be a reliable and potential marker for DFTD.

To further evaluate the diagnostic utility of this candidate, periaxin labelling was also tested in non-DFTD Tasmanian devil tumours and alternative models of the disease such as murine xenografted DFTD tumours, DFTD cell lines and devil Schwann cells in primary culture. Nine non-DFTD tumours were evaluated for the expression of periaxin. Samples included three mammary adenocarcinomas, three histiocytomas, one cystic papillary adenocarcinoma, one squamous cell carcinoma and one hepatic fibrosarcoma. None of the non-DFTD tumours were positive for periaxin (Figures 3.2.E-F). On the other hand, strong periaxin labelling was observed in all neoplastic cells within xenografted DFTD tumours (Figure 3.2.G-H). The periaxin labelling was maintained in tumours from over two passages.

Finally, immunofluorescence techniques were used to confirm the expression of periaxin in primary cultured Schwann cells from an adult Tasmanian devil and DFTD cultured cell lines. Periaxin expression was conserved in normal Schwann cells and was a useful marker to differentiate these cells from co-cultured fibroblasts (Figure 3.3.A-C). Periaxin also consistently labelled all DFTD tumour cells obtained from tissue culture (Figure 3.3.D-F).

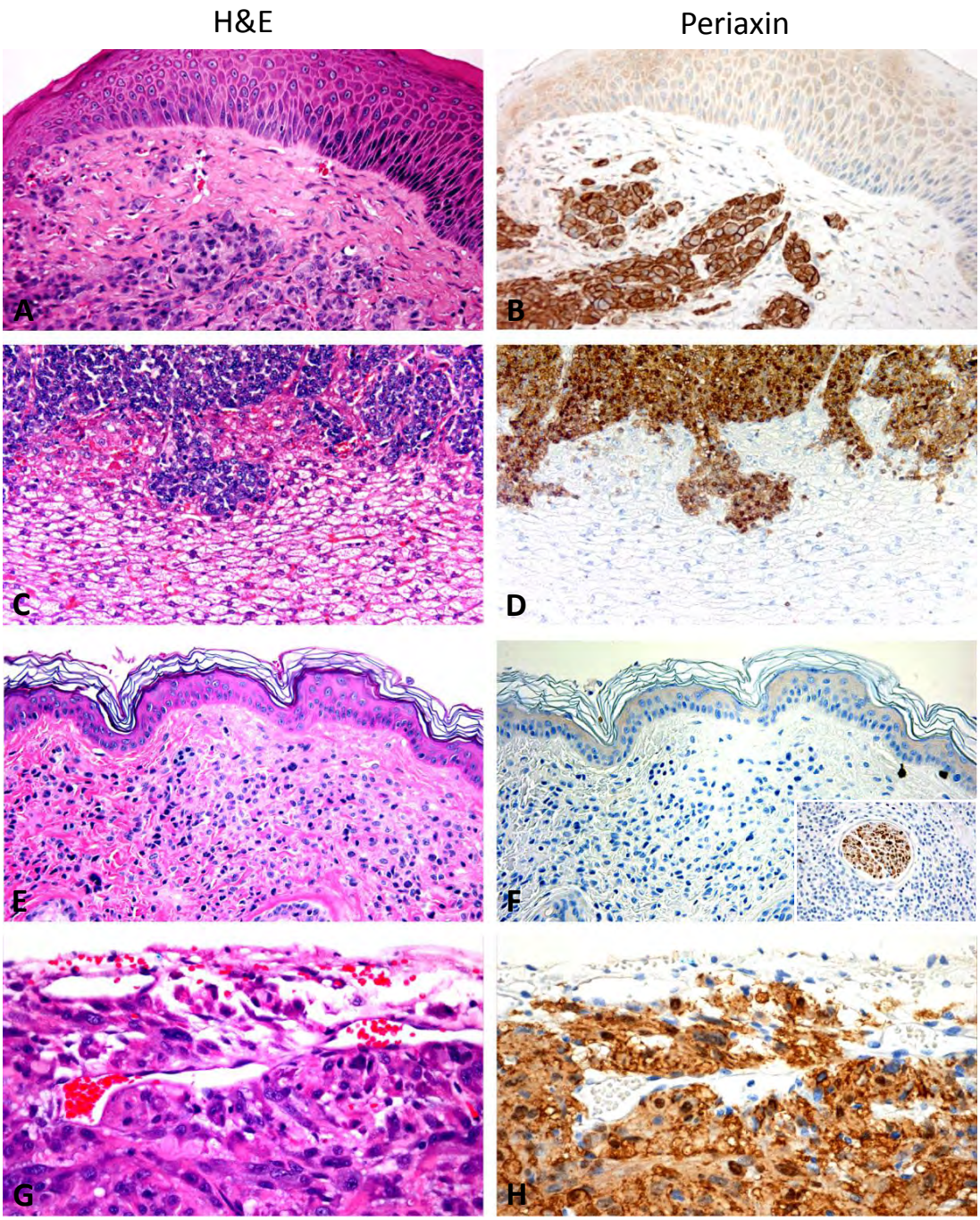


Figure 3.2 Periaxin expression in DFTD and non-DFTD tumours. **A,** DFTD primary tumours cells in the dermis. **B**, Strong and specific labelling of DFTD cells with periaxin. **C**, H&E staining showing the presence of a secondary DFTD tumour (dense cell aggregates) within the liver. **D**, DFTD tumour cells show strong immunohistochemical labelling for periaxin, which allows differentiation from the surrounding normal liver tissue. **E**, Devils are prone to a variety of neoplastic processes. This H&E staining shows adenocarcinoma cells (dense cell aggregates) in the dermis. **F**, Adenocarcinoma cells did not showed immunoreactivity for periaxin. However, periaxin labelled peripheral nerves in the tissue sample (insert). **G**, DFTD tumour induced in NOD/SCID mice by inoculation of DFTD cultured cells. Histological features are similar to DFTD tumours. **H**, Periaxin expression is present in all xenografted DFTD tumour cells. Left panels H&E staining. Right panels immunohistochemistry with EnVision+ system (Dako) detection and hematoxylin counterstain.

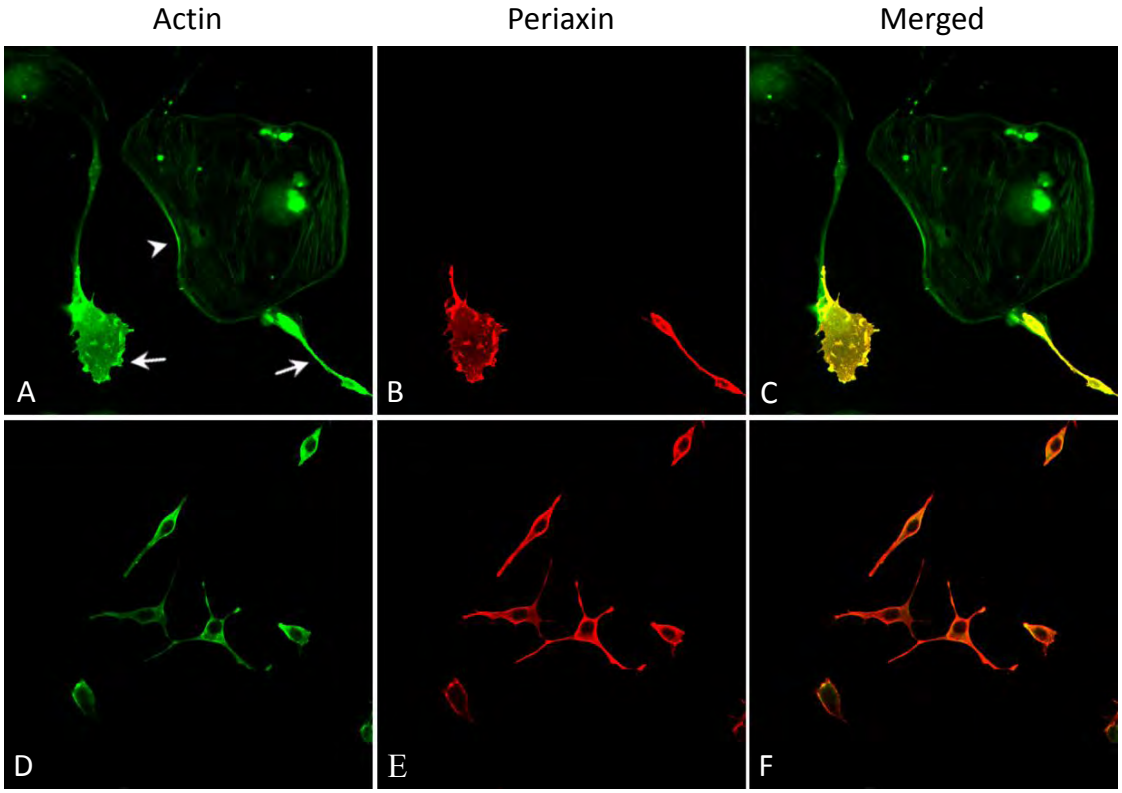


Figure 3.3 Expression of periaxin in primary cell culture of adult devil Schwann cells and DFTD cell lines. **A**, Primary Schwann cell cultures are normally contaminated by fibroblasts. Both Schwann cells (arrows) and fibroblasts (arrowhead) were detected with actin (green). **B**, Schwann cells in culture expressed periaxin (red), which allowed them to be differentiated from fibroblasts. **C**, Images A and B merged. DFTD cells in culture were dual labelled with actin (green, panel **D**) and periaxin antibody (red – panel **E**). As shown in the merged panel **F**, all DFTD tumour cells were positive for periaxin.

3.4 Discussion

The previous chapter showed evidence indicating that the devil facial tumour disease (DFTD) is a peripheral nerve sheath tumour (PNST) that arose from a Schwann cell. In this study, eight neuronal and peripheral nerve proteins were evaluated for their utility as diagnostic markers for DFTD. Proteins evaluated included periaxin, S100 protein, peripheral myelin protein 22, nerve growth factor receptor, nestin, neuron specific enolase, chromogranin A, and myelin basic protein. Of these, periaxin was confirmed as the most sensitive and specific marker, labelling the majority of DFTD cells in all primary DFTD tumours and DFTD metastases. In normal tissues, periaxin showed specificity for Schwann cells in peripheral nerve bundles. Periaxin expression was also maintained in cultured devil Schwann cells, DFTD cell lines, and xenografted DFTD tumours validating its utility as a diagnostic marker for the disease.

PNST tumours have been described in humans and in several animal models (Bundza *et al.*, 1986, Chijiwa *et al.*, 2004, Nielsen *et al.*, 2007, Schoniger and Summers, 2009, Schulman *et al.*, 2009). Diagnosis of these tumours still remains a diagnostic challenge due to the lack of specific immunohistochemical markers of neural differentiation (Sandberg, 2008). Although labelling for S100 protein (S100) shows some non-specific activity, it is the most commonly used marker to identify PNST of various types (Coindre, 2003, Wu and Montgomery, 2008). In this study, S100 expression was observed in all DFTD tumour samples. The intensity of labelling and fraction of immunoreactive cells varied considerably among samples. This is a common finding with other studies (Nonaka *et al.*, 2008, Stasik and Tawfik, 2006). S100 labelling was also observed in other tissues such as endothelium, skeletal muscle, hair follicles, kidney and connective tissue, and therefore is not a cell-specific marker.

Other markers normally tested in PNST with different levels of reliability include myelin basic protein (MBP), nerve growth factor receptor (NGFR) and nestin (Chijiwa *et al.*, 2004, Hoshi *et al.*, 1994, Shimada *et al.*, 2007, Stasik and Tawfik, 2006). NGFR positivity was found in 70% of the DFTD tumour samples. However, intensity of labelling was highly variable. Nestin and MBP stained only a small fraction of samples. Interpretation of results was also difficult due to the high level of non-specific labelling with MBP.

This study also tested periaxin, a marker of Schwann cell lineage (Gillespie *et al.*, 1994) that was found to be expressed in the devil transcriptome (Murchison *et al.*, 2010). The labelling for periaxin was intense and specific in DFTD tumours. Periaxin strongly labelled almost 100% of the neoplastic cells in all DFTD primary tumours with minimal non-specific labelling.

Remarkably, the high levels of periaxin protein expression were maintained in all DFTD metastases. Protein expression was also preserved after the xeno-transplantation and passage of DFTD tumour cells into NOD-SCID mice, including tumours from the second generation. Similarly, cultured devil Schwann cells and DFTD tumour cell lines were all positive for periaxin. These results provide evidence for the genetic stability of periaxin *in vivo* and *in vitro* and reinforce the value of the protein as a diagnostic marker in a wide range of DFTD models.

In contrast, non-DFTD tumours did not show periaxin expression. Consequently, periaxin expression is useful for the differentiation of DFTD tumours from other cancers of similar appearance. Similarly, periaxin can be a useful marker for identifying tumour cells in early DFTD tumours. These small tumours can be difficult to distinguish from the lacerations and scars often observed in the face of the animals, which are produced by the common biting behaviour in devils (Hamede *et al.*, 2008). Fixed sample from small punch biopsies are useful for this purpose.

Given the clonal and stable characteristics of the DFTD karyotype (Deakin *et al.*, 2012), current gold standard for tumour identification is cytogenetics. The distinctive karyotype is specific for devil facial tumour cells and is an important tool for diagnosis and monitoring the evolution of the disease (Deakin *et al.*, 2012, Pearse and Swift, 2006). One of the major disadvantages is the need for fresh tumour samples for establishment of cell cultures under sterile conditions before chromosome analysis (Sandberg and Meloni-Ehrig, 2010). This is time consuming and labour intensive. Periaxin offer a reliable and quicker alternative.

Therefore, this thesis proposes periaxin as a reliable, sensitive and specific marker for DFTD. It has been suggested that the most valuable immunohistologic markers are those that are linked to the cell of origin or those that reflect the genetic signature of the

tumour (Natkunam and Mason, 2006). As shown here, periaxin certainly meets these two criteria.

The diagnostic of PNST in humans relies in S100 expression plus a range of other unreliable markers (Coindre, 2003). This thesis showed that periaxin was consistent and specifically expressed in DFTD, which is a PNST. The study of DFTD may have relevance for human cancer by identifying a reliable diagnostic marker.

An interesting aspect of the findings is that despite their similar morphology, the tumour cells surrounding the tumour nests have higher levels of protein expression, especially in the areas where the cells are in close contact with the surrounding normal connective tissue. The role of the tumour microenvironment in tumour growth and invasion has been widely investigated (Albini and Sporn, 2007, Bierie and Moses, 2006). It is probable that the interactions between the cancer cells at the periphery of the tumour nest and the surrounding stromal cells induce differential protein expression compared to that of the internal tumour cells. The variability of expression of the markers between, and within, tumours could also reflect different stages of tumour development. This could be revealed in the current study by the pattern of growth of the tumour fascicles or nodules, which is clearly concentric and layered with centralized areas of tumour cell necrosis. The interaction of DFTD tumour cells with the extracellular matrix and its function for tumour progression is an important aspect that will require further investigation.

In conclusion, this study presents periaxin as a new sensitive marker for DFTD that will greatly facilitate the diagnosis of the disease in histological and cytological samples. Periaxin expression is maintained in DFTD metastases, xenografted tumours and cell lines supporting its importance as a biomarker for the disease. Using immunohistochemistry techniques for diagnosis of DFTD in fixed tissue samples has evident advantages compared to the current cytogenetics methods. Periaxin could also be a potential marker for the diagnosis of neoplasias of Schwann cell origin in other species. Thus, the improvement of the diagnosis of DFTD and the finding that over expressed genes in the tumour (Murchison *et al.*, 2010) can be recognized with specific markers open new opportunities for the understanding and management of this unusual disease.

4 Analysis of MHC class I expression in DFTD

Table of Contents

4.1 Introduction

Devil facial tumour cells spread between unrelated individuals without evoking an immune response despite the devils having a competent immune system (Kreiss *et al.*, 2008, Kreiss, 2009, Kreiss *et al.*, 2009, Woods *et al.*, 2007). Affected animals die within months of tumour appearance and the species could face extinction within 25 years (Hawkins *et al.*, 2006).

Initial sequencing analyses showed that devils have low levels of genetic diversity at the major histocompatibility complex (MHC) (Siddle *et al.,* 2007b) and devils from eastern Tasmania showed weak responses to allogeneic mixed lymphocyte reactions (MLR) in culture (Woods *et al.,* 2007). This combined data suggested that DFTD tumour cells and the host might be identical at the MHC loci allowing the tumour to spread without encountering any histocompatibility barriers (Siddle *et al.,* 2007a, Siddle *et al.,* 2007b).

Experimental transplantation of skin grafts and MLR between unrelated devils provided evidence that the devils are capable of allogeneic responses (Kreiss *et al.*, 2011a). Devils rejected the skin grafts regardless of the MHC type and the strongest MLR responses occurred when mixing lymphocytes from eastern Tasmanian devils with lymphocytes from western Tasmanian animals. Therefore, it is likely that DFTD evolved special adaptive mechanisms that allow allotransplantation of tumour cells without immune recognition.

Downregulation of MHC class I (MHC-I) expression is very common in cancer (Aptsiauri *et al.*, 2007). This particular mechanism allows successful allotransplantation of cancer cells in the canine transmissible venereal tumour (CTVT), the only other naturally occurring contagious cancer (Cohen *et al.*, 1984). However, MHC-I expression in devil facial tumour cells has only been studied at the genetic level. Thus, this chapter investigated the protein expression of MHC-I to determine if this mechanism may explain the lack of immune response to DFTD cells.

As specific anti-devil reagents were not available, cross-reactivity of a range of commercial anti-MHC antibodies were initially evaluated. Anti-human, anti-mouse and anti- short-tailed opossum (*Monodelphis domestica*) antibodies developed and provided by research collaborators, were also evaluated. An anti-devil MHC-I antibody recently

developed by collaborators in UK was also tested in this current study. A wide range of techniques were used to evaluate MHC-I protein expression. These included flow cytometry of DFTD cell lines and immunofluorescence performed on DFTD cell lines and devil primary cultured fibroblasts. DFTD primary tumours and metastases were also evaluated using immunohistochemistry. Western blot was performed using protein extraction from primary tumours, devil tissues and cell lines.

4.2 Methods

4.2.1 Flow Cytometry

Human, Tasmanian devil and opossum leukocytes isolated from peripheral blood were used to test the suitability and cross-reactivity of readily available anti-MHC class I antibodies.

Approximately 10 ml of blood was taken from the jugular vein from anaesthetised Tasmanian devils (permit # A0009215 from the University of Tasmania Animal Ethics Committee) following standard veterinary procedures previously described (Brown *et al.*, 2011). Approximately 5 ml of blood was collected during autopsy of captive opossums (held at Melbourne University). Approximately 10 ml of human blood samples were taken from volunteer donors (permit # H0011494 from the University of Tasmania Human Research Ethics Committee). Blood samples were collected in heparin tubes and kept at room temperature. Under sterile conditions, each sample was diluted 1:1 with PBS. In 15 ml centrifuge tubes, the diluted blood samples were layered onto a Histopaque 1077 gradient (Sigma, USA) and then centrifuged at 400 × *g* for 30 minutes. The mononuclear cell layer was collected from each sample into a separate centrifuge tube, 10 ml PBS added and centrifuged at 250 × *g* for 10 minutes to wash. The wash step was then repeated. Samples were then resuspended in PBS containing 10% foetal calf serum. Appendix 2 provides a complete list of reagents.

MHC-I expression was assessed with flow cytometry using a wide range of readily available anti-MHC class I antibodies in order to examine potential inter-species cross-reactivity (Table 4.1). All assays were run in triplicate. The flow cytometry protocol was as described in chapter 2.2.4. Briefly, cell surface MHC-I expression was assessed in intact (non-permeabilized) leukocytes labelled with primary antibody and an Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Fluorescence intensity was detected in a BD FACS canto II flow cytometer and analysed using BD CellQuest Pro v5.2.1 software. For analysis of MHC-I intracellular expression, the leukocytes were first fixed and permeabilized, using Fix and Perm Cell Permeabilization Reagents GAS003 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

4.2.2 Western blot

Protein samples

Samples of primary DFTD tumours, normal devil tissues and murine xenografted DFTD tumours were supplied by the tissue bank held at the Menzies Research Institute Tasmania, University of Tasmania. Tissues samples were kept at -80 ^{o}C. DFTD cell lines were provided by the Department of Primary Industry, Parks, Water and Environment (DPIPWE), Tasmania.

Table 4.1 List of anti-MHC-I antibodies tested against devil peripheral blood mononuclear cells

Antibody	Species	Reactivity	Antibody	Species	Reactivity
Commercial MHC-I 2G5 *AbD Serotec MCA2189*	Mouse IgG2b	Rat, Guinea pig, sheep, bovine, pig, human, hamster	MHC-I EH-144*	Mouse	Mouse
Commercial B2M*Abcam ab27588*	Mouse IgG2a	Human, pig	MHC-I Y-3*	Mouse	Mouse
MHC-I W6/32*	Mouse	Human	MHC-I 34.7.23*	Mouse	Mouse
MHC-I Y25*	Mouse	Mouse	MHC-I MKD6*	Mouse	Mouse
MHC-I 5FI*	Mouse	Mouse	MHC-I 20.8.4*	Mouse	Mouse
MHC-I 16.12*	Mouse	Mouse	MHC-I 28.11.5*	Mouse	Mouse
MHC-I BW4*	Mouse	Human	MHC-I 3.4.2.12*	Mouse	Mouse
MHC-I 2.28*	Mouse	Human	MHC-I B721*	Mouse	Human
MHC-I BB7.7*	Mouse	Human	MHC Modo UA**	Rabbit	Opossum
MHC-I B27*	Mouse	Human	MHC Modo UG**	Rabbit	Opossum
MHC-I 20C3*	Mouse	Human	MHC Modo UJ**	Rabbit	Opossum
MHC-I 3E12*	Mouse	Human	MHC-I(TD5, TD26, TD35, TD50)***	Mouse	Devil
MHC-I 20F2*	Mouse	Human			

Commercial antibodies were used at 1/200 dilution. *Culture supernatant provided by J. McCluskey (University of Melbourne) were used at 1/3 dilution. **Antibodies (purified rabbit serum) developed and provided by M. Baker (University of New Mexico, USA) and used at 1/100 dilution. ***Antibodies (supernatant) developed and provided by H. Siddle and J. Kaufman (University of Cambridge, UK). TD5, TD26, TD35 and TD50 represent four clones against the synthetic peptide (GGKGGDYVPAAGN) representing the cytoplasmic tail of devil MHC-I; each clone was used at 1/50 dilution.

Total protein was extracted from devil tissues, DFTD tumours, leukocytes and cell lines using 1 ml of ice-cold RIPA buffer (Thermo Scientific, Rockford, IL) containing 10 µl of Halt™ protease inhibitor cocktail (Thermo Scientific) for approximately 40 mg of tissue sample or cell pellet. Sample homogenization was performed in 2 ml screw-cap vials containing 1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) and extraction buffer. Total disruption of the tissue was obtained in 4-5 shaking cycles of 1 minute in a Mini-bead beater model 607 (BioSpec Products). The sample was then centrifuged at 14,000 × *g* for 30 minutes at room temperature and the supernatant transferred to a new tube for protein concentration quantification using the DC-protein assay (Bio-Rad, Hercules, CA) following manufacturer's specifications.

SDS-electrophoresis

Electrophoresis was performed using the NuPAGE® system and the XCell SureLock™ mini cell (Invitrogen, Carlsbad, CA).

40 µg of protein sample was added to NuPAGE® LDS sample buffer (4X) and NuPAGE® reducing agent (10X) with the remaining volume made up by Milli-Q water to either 10 µl or 20 µl depending on the protein concentration. The samples were vortexed, centrifuged briefly and heated at 70 ^{o}C for 10.

Samples were loaded on 4-12% NuPAGE® Bis-Tris mini-gels. One of the lanes was loaded with a molecular weight marker (Spectra Multicolor Broad Range Protein Ladder – Thermo Scientific). The inner buffer chamber of the mini-cell was filled with 200 ml of NuPAGE® SDS MOPS running buffer containing 500 µl of NuPAGE® antioxidant. The outer buffer chamber was filled with 600 ml of NuPAGE® SDS MOPS running buffer. The gel was run at 200 V for 50 minutes.

Protein transfer

Electroblotting of proteins from the gel to a nitrocellulose membrane, 0.45 µm (Bio-Rad, Germany) was performed using the XCell II™ Blot Module system (Invitrogen). Blotting was performed using 1XNuPAGE® transfer buffer with 10% methanol overnight at 4 ^{o}C and 25 V.

Immunodetection

Membranes were blocked in TBSTM (TBS buffer, 0.1% Tween 20, 5% skim milk) for 1 hour at room temperature. The membrane was then incubated with the primary antibody diluted in TBSTM for 2 hours at room temperature, quickly washed twice with TBST (TBS, 0.1% Tween 20) and then 4 × 5 minutes with TBST at room temperature. The membranes were then incubated with the horseradish peroxidase (HRP)-conjugated anti-rabbit (NIF 824) or anti-mouse (NIF 825) secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted in TBSTM for one hour and again washed as described above. Chemiluminescent detection was performed following membrane immersion with Immobilon™ Western HRP substrate (Millipore, Billerica, MA) for 5 minutes at room temperature using a Chemi-Smart 5000 digital camera (Vilber Lourmat, EEC). Amersham

(Piscataway, NJ)

4.2.3 Immunohistochemistry

Samples from primary DFTD tumours, DFTD metastases and normal tissues were evaluated by immunohistochemistry for the expression of MHC-I. Serial sections were used to identify DFTD cells and tumour nests by their labelling with the anti-periaxin antibody (Sigma/HPA 001868). Tissue section preparations and immunohistochemistry protocols were as described in Chapter 2.2. Optimal concentration of the anti-devil MHC-I antibody was initially determined by serial dilution. The preferred concentration for each clone was 1/50.

4.2.4 Immunofluorescence

Cell lines

DFTD cell lines provided by the DPIPWE were plated onto Poly-L-Lysine (Sigma)-coated coverslips and incubated to semi-confluence at 37 ^{o}C and 5% CO_2 in complete RPMI medium (RPMI 1640, Gibco, New Zealand) containing 10% foetal calf serum, 2 mM of L-glutamine (Sigma) and 40 mg/ml of gentamicin (Pfizer, Australia).

Primary fibroblasts cell culture

In order to compare the MHC-I expression of DFTD tumour cells with that of normal cells, a primary culture of fibroblast was established. Samples of ovary and uterus tissue were taken during the autopsy of an adult devil. The tissue was dissociated with a sterile scalpel in PBS containing 0.25% trypsin (Sigma) and incubated overnight at 4 ^{o}C. The tissue was then diluted with 3 ml of complete RPMI medium and washed through a 70 µm cell strainer (BD Falcon, Franklin Lakes, NJ) with 3 ml of complete RPMI medium. The filtrate was centrifuged at 500 × *g* for 3 minutes and the supernatant discarded. The cell pellet was resuspended in 8 ml of complete RPMI medium, plated onto Poly-L-Lysine-coated coverslips and incubated to semi-confluence at 37 ^{o}C and 5% CO_2.

Primary DFTD cells culture

One to two needle aspiration biopsies (approximately 200 ul each) were taken from a primary DFTD tumour and collected in complete RPMI medium. The cell suspension was washed through a 70 µm cell strainer and the filtrate centrifuged at 500 × *g* for 3 minutes. The cell pellet was resuspended in 8 ml of complete RPMI medium, plated onto Poly-L-Lysine-coated coverslips and incubated to semi-confluence at 37 ^{o}C and 5% CO_2. The same procedure was repeated with at least three tumours from different individuals and the best culture selected for further analysis.

Immunofluorescence

Immunofluorescence protocol was as described in section 3.2.4. Briefly, cells growing on coverslips were fixed with paraformaldehyde before labelling with the primary antibodies (mouse anti-devil MHC-I and rabbit anti-periaxin) and secondary antibodies (Alexa Fluor 488 goat anti-mouse, Alexa Fluor 633 goat anti-rabbit [Invitrogen]). Actin stain (Alexa Fluor 546 phalloidin, Invitrogen) was also added to the cells as a counterstain and to visualise all the cells. A confocal microscope was used for visualization and imaging of the labelling.

4.3 Results

Flow cytometry, western blotting, immunohistochemistry and immunofluorescence techniques were used to study the expression of MHC molecules in devil tissues and DFTD cells. Due to the lack of specific antibodies against devil MHC molecules, a significant part of the research involved testing a broad range of commercial reagents and in house designed reagents provided by research collaborators. This section also presents a summary of these screening studies.

4.3.1 Screening the reactivity of readily available anti-MHC-I antibodies

Flow cytometry was used to screen and test the reactivity of commercial and other available antibodies against MHC-I molecules on devil peripheral blood mononuclear cells. These included antibodies widely used such as the clone W6/32 which is conformationally dependent on the expression of both MHC-heavy chain and beta-2-microglobulin (B2M); the clone 2G5, a conformationally dependent antibody that recognises an MHC-I epitope phylogenetically conserved, and an antibody against the highly conserved B2M. In total 19 different antibodies against human and mouse MHC-I donated by colleagues at the University of Melbourne (Australia) were tested. All antibodies were tested in triplicate.

This section presents representative results showing only three of the antibodies tested, the three that were most likely to display cross-reactivity with devil MHC-I (i.e. the clones W6/32, 2G5 and B2M). MHC-I expression at the cell surface (intact cells) and intracellular (permeabilized cells) was clearly detected in the human leukocytes used as positive control. However, no cross-reactivity was observed with the three antibodies tested in devil leukocytes (Figure 4.1). All the remaining 18 antibodies were also negative with devil leukocytes.

Colleagues at the University of New Mexico (USA) developed polyclonal antibodies against published classical (Modo UA) and non-classical (Modo UG, Modo UJ) MHC-I sequences of the grey, short-tailed opossum (*Monodelphis domestica*), a South American marsupial (Baker *et al.*, 2009, Gouin *et al.*, 2006, Miska and Miller, 1999). The specificity of these antibodies was assessed for the first time in both opossum and Tasmanian devil leukocytes. The antibodies were tested in permeabilized and non-

permeabilized cells using flow cytometry (Figure 4.2). Unfortunately, these antibodies failed to detect MHC-I in the intact opossum leukocytes that were used as a positive control. Modo UG (a non-classical MHC-I molecule) was detected in permeabilized opossum leukocytes although high background was also observed with the isotype control. The non-classical Modo UJ was also detected but at the same level as the isotype. Therefore, the reactivity of these antibodies for detection of devil MHC-I was difficult to interpret and they were not considered useful for further analyses.

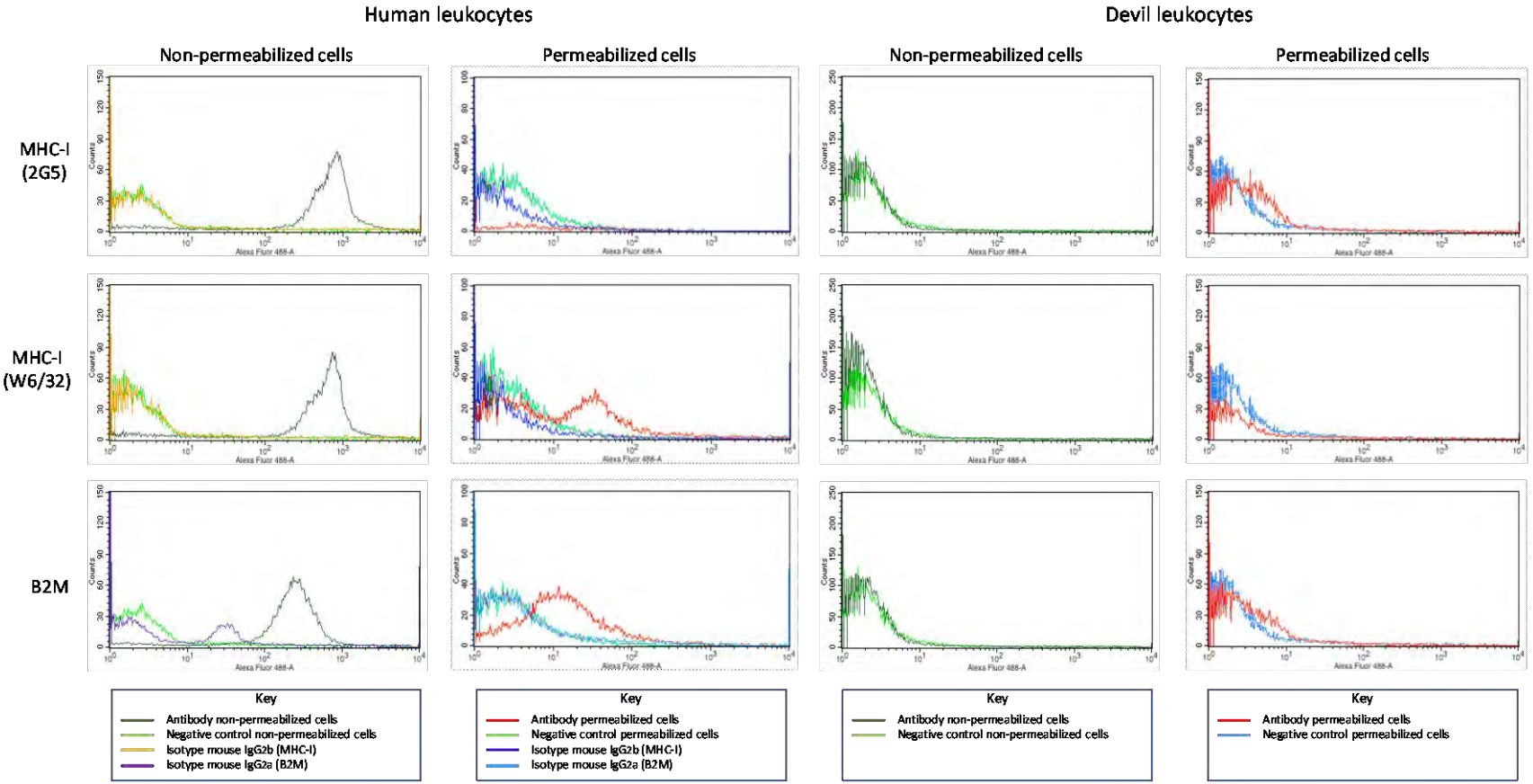


Figure 4.1 Studies of tissue cross-reactivity of readily available MHC-I antibodies on Tasmanian devil leukocytes. Two antibodies against MHC-I (clone 2G5 and clone W6/32) and one antibody against beta-2-microglobulin (B2M) were tested on human and devil leukocytes isolated from peripheral blood. Cell surface or intracellular MHC-I expression was investigated in non-permeabilized or permeabilized cells respectively using flow cytometry. Cell surface MHC-I expression was clearly observed in the non-permeabilized human leukocytes used as a positive control (left panels). Intracellular expression was also detected with the clone W6/32 and the B2M antibody in human leukocytes. In contrast, these antibodies failed to detect surface or intracellular MHC-I expression in devil leukocytes (right panels). Isotype and negative controls (omitting the primary antibody) are also shown.

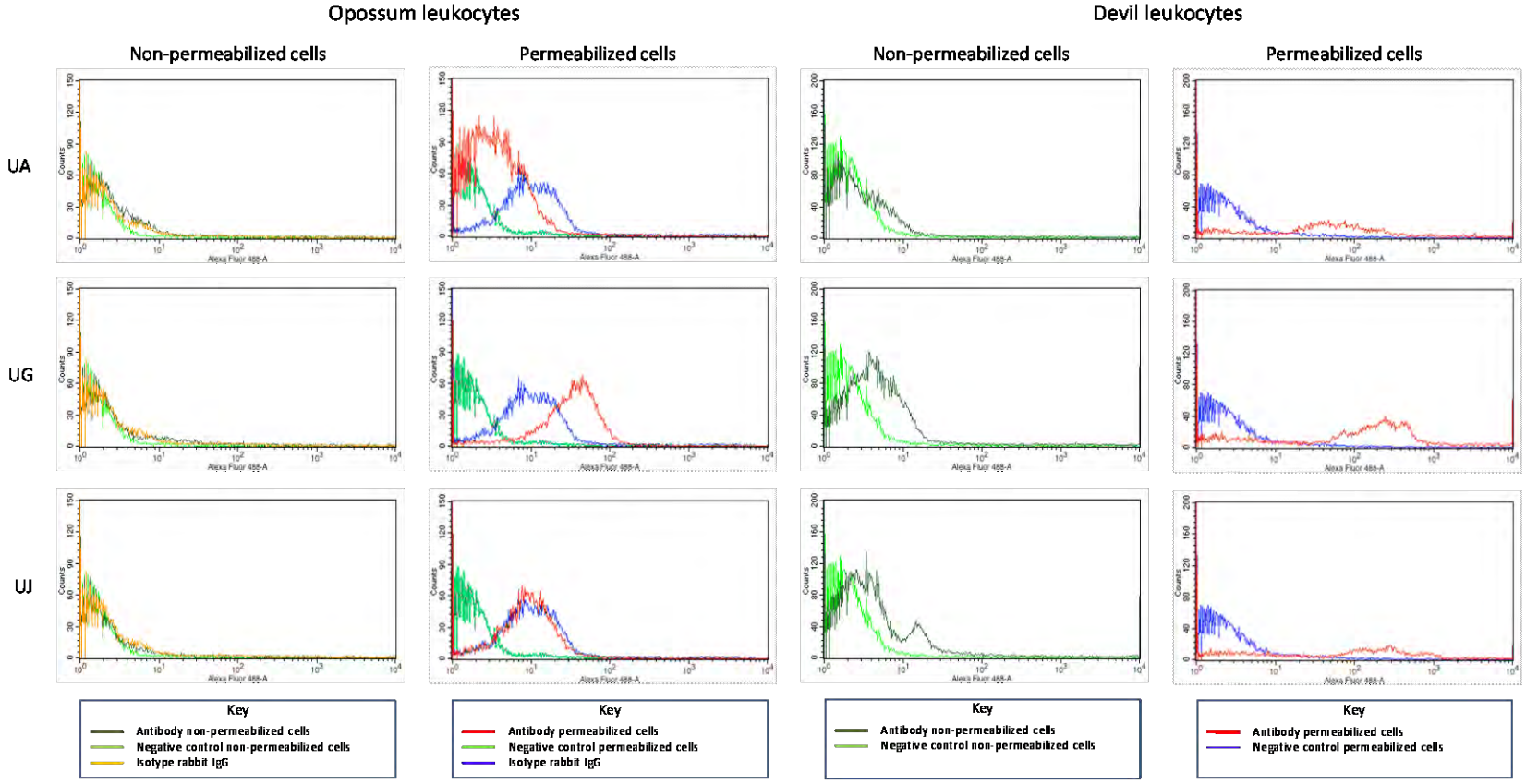


Figure 4.2 Studies of tissue cross-reactivity of anti-opossum MHC-I antibodies on opossum and Tasmanian devil leukocytes. Three anti-opossum MHC-I antibodies against classical (modo UA) and non-classical molecules (modo UG and modo UJ) were tested on opossum and devil leukocytes isolated from peripheral blood using flow cytometry. Cell surface or intracellular MHC-I expression was investigated in non-permeabilized or permeabilized cells respectively. No cell surface expression was detected in opossum leukocytes used as positive control. High levels of background (as determined by the isotype control) were also observed in the permeabilized opossum leukocytes (left panels). The lack of surface reactivity and high levels of background in the positive controls make the interpretation of the results in the devil leukocytes difficult and uncertain (right panels). Thus, the antibodies were not considered suitable for further studies.

4.3.2 Assessing the reactivity of custom-designed monoclonal anti-devil MHC-I antibodies

During the course of this research, H. Siddle and J. Kaufman, collaborators from the University of Cambridge (UK), developed the first monoclonal antibodies against a synthetic peptide (GGKGGDYVPAAGN) representing the cytoplasmic region of a devil MHC-I sequence (Siddle *et al.*, 2007b). The antibodies included four clones (from the same exon) designated as TD5, TD26, TD35 and TD50. Western blot analyses were used to test the specificity and suitability of these antibodies for detection of MHC-I in proteins isolated from tissue samples. Testing the clones individually, MHC-I was detected in proteins extracted from devil spleen, kidney and leukocytes. Specificity was demonstrated by detection of a single band of approximately 40 kDa (the molecular weight expected for the MHC-I molecule) in the three tissue samples. Results were consistent for the four antibodies (Figure 4.3.A). Spleen and leukocytes showed similar levels of expression and therefore these tissues were selected as positive controls for further experiments.

Specificity of the antibodies was also confirmed using mouse IgG as an isotype control and omitting the primary antibody as a negative control. In this experiment the four MHC-I clones were combined as an antibody cocktail without altering the results previously observed (Figure 4.3.B). This preparation (combining the four clones) was then used for further experiments.

Finally, commercial antibodies against the cytoskeleton protein actin (beta actin polyclonal antibody ab729, Abcam, Cambridge, UK), the microtubule protein tubulin (alpha tubulin monoclonal antibody ab8227, Abcam) and the glycolytic enzyme phosphoglycerate kinase 1 (PGK1 monoclonal antibody ab67335, Abcam) were tested as loading controls for the western blot analyses. Cross-reactivity of these antibodies was assessed in proteins isolated from devil spleen tissue and primary devil facial tumours. DFTD tumour showed higher levels of expression of PGK1 and tubulin compared to spleen. The level of expression of actin was similar in both tissues (Figure 4.3.C) and thus it was selected as the loading control. Actin has a close molecular weight to that of the MHC-I (around 40 kDa) making it difficult to detect both proteins in one single lane. Therefore, actin controls were loaded in a separated lane and run in parallel under the same conditions.

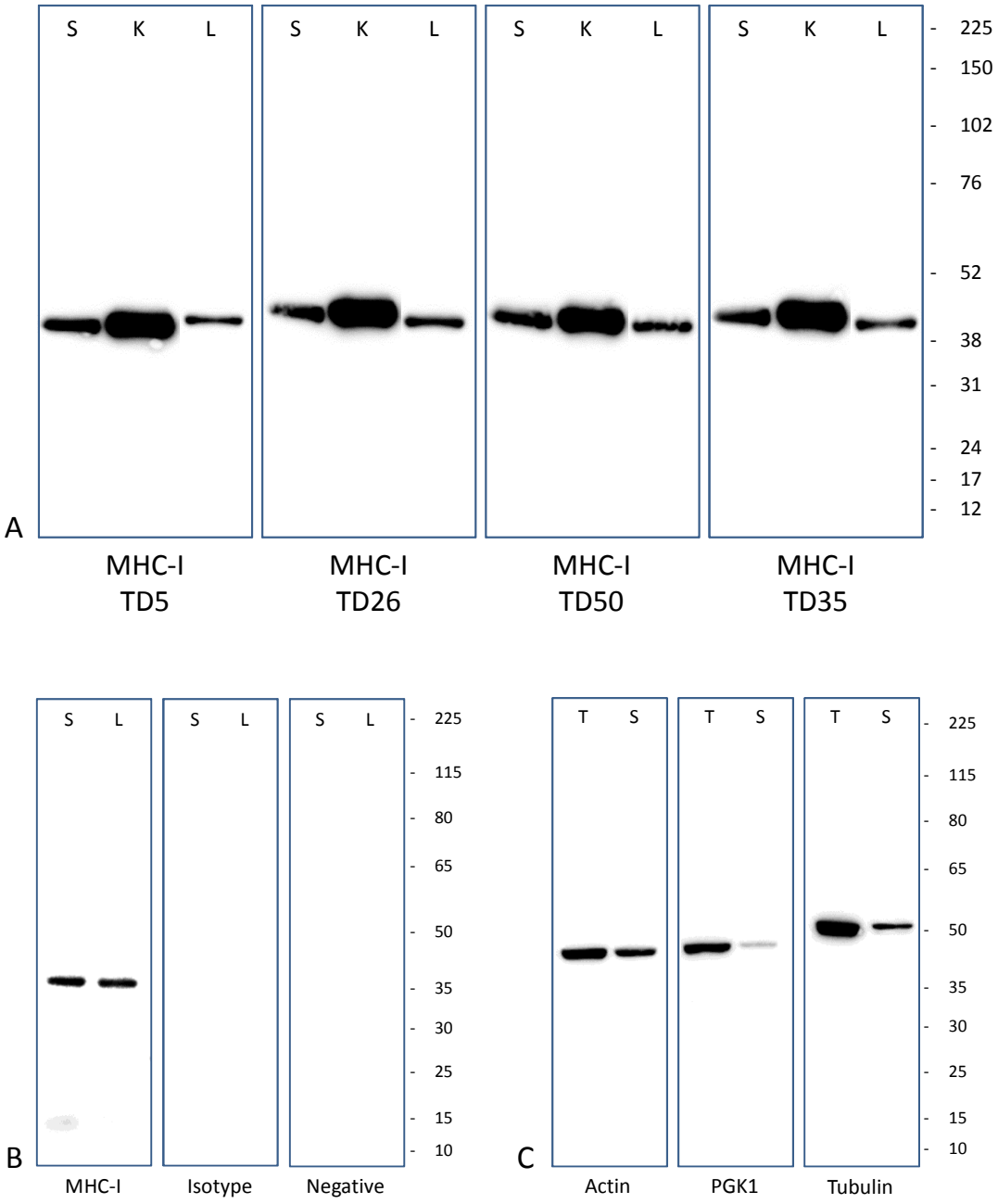


Figure 4.3 Testing the suitability of anti-devil MHC-I antibodies for protein expression analyses in devil tissues using western blotting. **A,** Total protein (40 µg) extracted from devil spleen (S), kidney (K) and leukocytes (L) was separated using gel electrophoresis and transferred onto nitrocellulose membranes for immune detection using custom antibodies against devil MHC-I (clones TD5, TD26, TD50, TD35). A single band was detected in the three tissues at 40 kDa, the molecular weight expected for the MHC-I molecule. **B,** Specificity of the custom antibodies was confirmed in protein extractions of devil spleen (S) and leukocytes isolated from peripheral blood (L). The membranes were probed with MHC-I antibody (a combination of the four clones), and isotype control (mouse IgG) or omitting the primary antibody (negative control). **C,** Three commercial loading controls were tested on proteins extracted from primary DFTD tumour (T) and devil spleen (S). Actin showed a more similar level of expression in both tissues compared to that of the phosphoglycerate kinase 1 (PGK1) and tubulin. Signal detection was achieved using a chemiluminescence substrate (Millipore) and images taken with a Chemi-Smart 5000 digital camera (Vilber Lourmat). Scale (molecular weight) at the right of the panels is in kDa.

4.3.3 Analysis of MHC-I expression in devil tissues and DFTD: Western blot analyses

MHC-I expression in normal tissue and primary DFTD tumours

The level of expression of MHC-I in normal devil tissue and primary DFTD tumours was assessed using western blot analysis as described previously. MHC-I expression was clear and consistently detected in all samples (10/10) of normal devil spleen. Four spleen samples had particularly high levels of expression (Figure 4.4). The devil spleen is a structurally complex organ with a capsule rich in blood vessels and a white pulp rich in lymphoid tissue. Random small tissue samples (around 0.5 cm^3) were taken during autopsy and it is likely that differences in MHC-I expression simply reflect the heterogeneity of the tissue.

MHC-I expression was clearly detected in seven of the fourteen DFTD tumour samples. Levels of expression in the positive samples were similar to that of the spleen. No expression, or low level of expression, was observed in the other seven DFTD samples. Additionally, some non-specific bands (approximately 60 kDa), not previously observed in normal tissues (kidney, spleen or leukocytes), were apparent in some of the tumour samples (Figure 4.4).

The loading control actin showed similar levels of expression in all samples of devil spleen and most DFTD tumours. This reinforced the observation that the levels of MHC-I expression are characteristic of the tissues and not due to different amounts of protein loaded for each sample.

MHC-I expression in DFTD cell lines and mouse xenografted DFTD tumours

MHC-I expression was weakly detected in proteins extracted from only one of the six DFTD cell lines. However, the level of MHC-I expression was lower compared to that of the spleen or the level of expression previously observed in isolated leukocytes (Figure 4.5). No bands were detected at the expected molecular weight for MHC-I in the other five DFTD cell lines. Instead, a band was detected at a molecular weight of approximately 60 kDa, similar to the additional band observed in proteins from primary DFTD tumours. Likewise, a band of similar size was detected in proteins extracted from

the cell line K562, a human leukaemia cell line that lacks MHC-I expression (Figure 4.5).

MHC-I expression was also assessed in proteins extracted from DFTD tumours that were xenografted onto NOD/SCID mice. MHC-I expression was not detected in any of the samples (9/9). The devil spleen sample loaded as a positive control showed the expected band (40 KDa) for MHC-I. Similar to the cell lines and primary DFTD tumours, a band around 60 kDa was detected in seven of the DFTD xenografts (Figure 4.5).

MHC-I expression in devil peripheral nerve tissue

Devil peripheral nerve, a tissue enriched with Schwann cells, was used to study the levels of MHC-I expression in these cells. After removal of the gross epineurial connective tissue whole cell proteins were extracted from adult devil peripheral nerves and analysed by western blot. No MHC-I expression was observed in any of the seven peripheral nerve protein samples analysed (Figure 4.6).

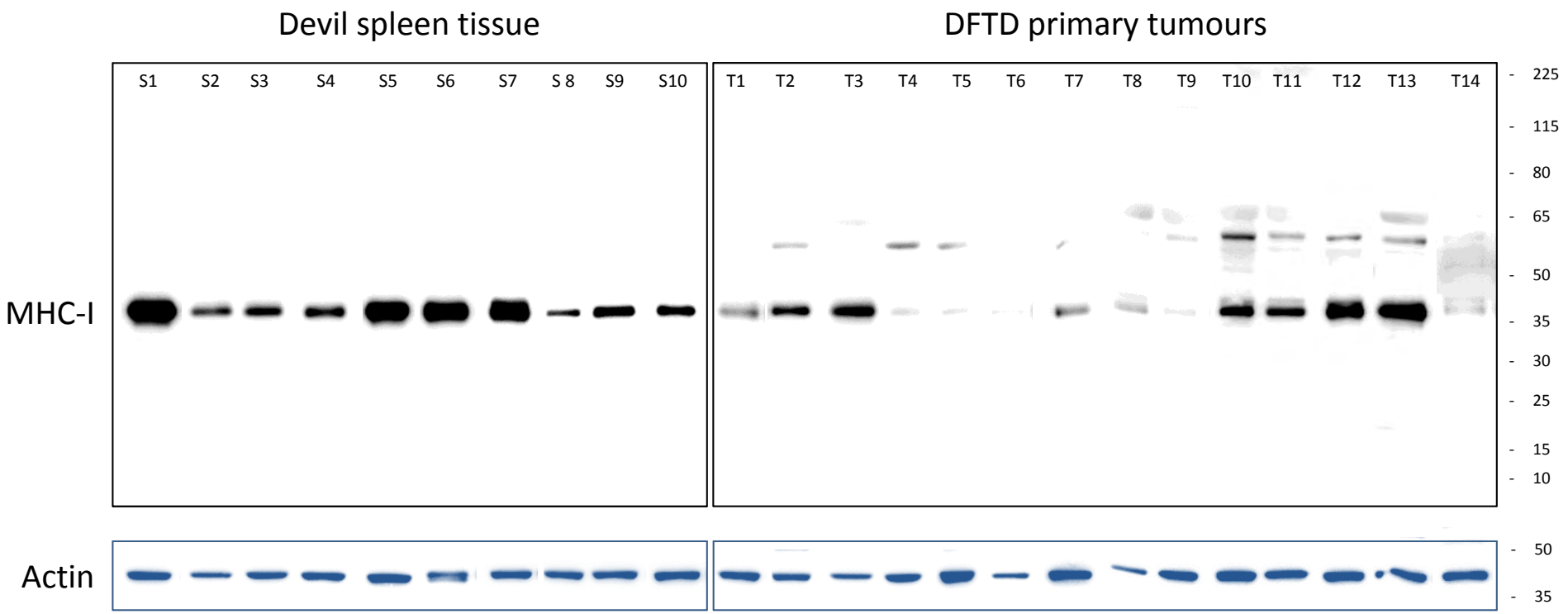


Figure 4.4 MHC-I expression in normal devil tissues and DFTD primary tumours. Western blots of total proteins (40 µg) from devil spleen (S1-S10) and DFTD primary tumours (T1-T14) probed with a cocktail of anti-devil MHC-I antibodies (clones TD5, TD26, TD35 and TD50). MHC-I was detected in all spleen samples as a unique band at 40 kDa (left panel). MHC-I was only clearly detected in seven of fourteen samples of DFTD primary tumours. An additional band at approximately 60 kDa was also observed in some of the tumour samples. Loading control actin is showed at the bottom of the panels. Signal detection was achieved using a chemiluminescence substrate (Millipore) and images taken with a Chemi-Smart 5000 digital camera (Vilber Lourmat). Scale (molecular weight) at the right of the panels is in kDa.

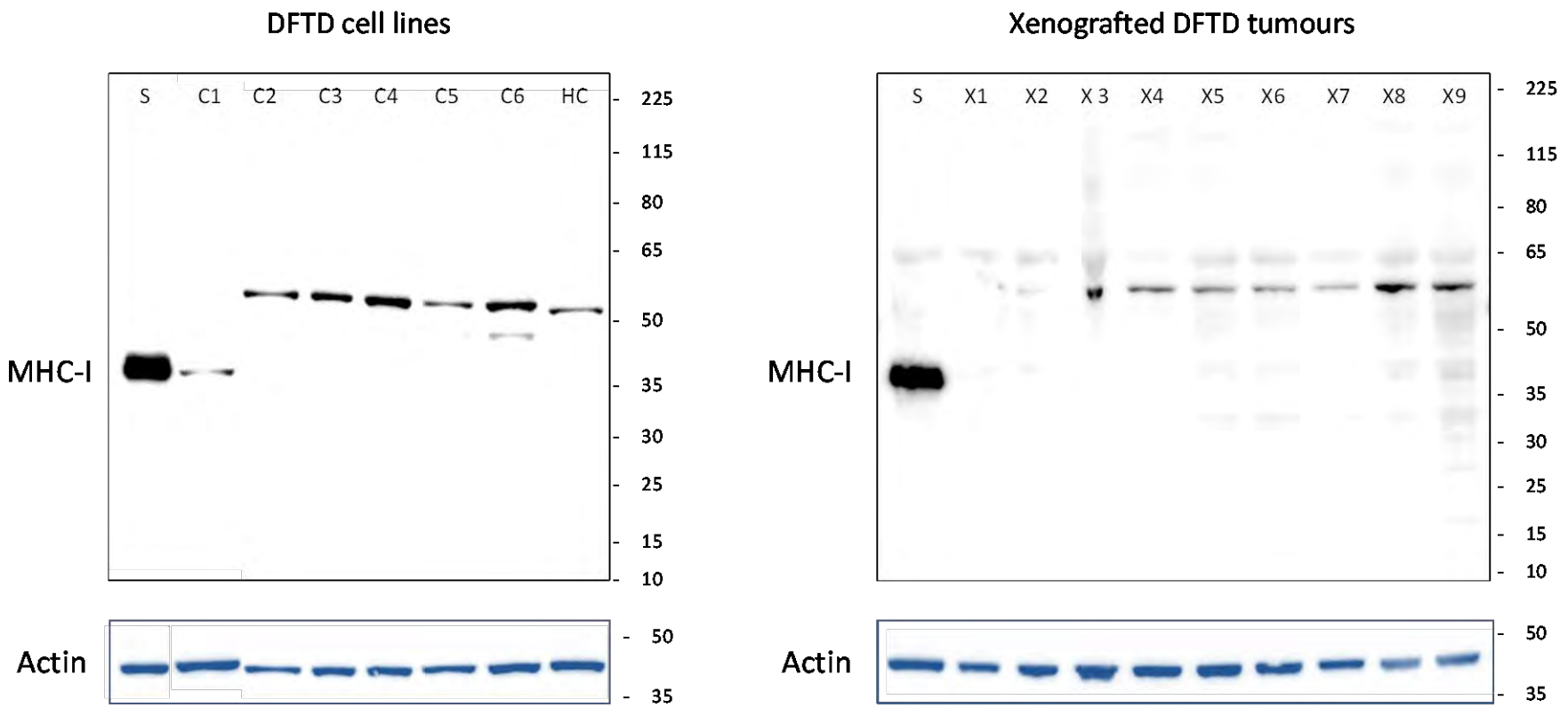


Figure 4.5 MHC-I expression in DFTD cell lines and murine xenografted DFTD tumours. Western blots of proteins extracted from DFTD cell lines (C1-C6) and xenografted DFTD tumours (X1-X9) probed with a cocktail of anti-devil MHC-I antibodies (clones TD5, TD26, TD35 and TD50). MHC-I was detected in only one cell line (left panel). None of the xenografted DFTD tumours showed MHC-I expression at the correct molecular weight. MHC-I was detected in the spleen sample (S) used as positive control. A band around 60 kDa was observed in most of the DFTD cell lines and xenografted tumours. A similar band was detected in the human leukaemia cell line K562 (HC in the left panel). Loading control actin is shown at the bottom of the panels. Signal detection was achieved using a chemiluminescence substrate (Millipore) and images taken with a Chemi-Smart 5000 digital camera (Vilber Lourmat). Scale (molecular weight) at the right of the membranes is in kDa.

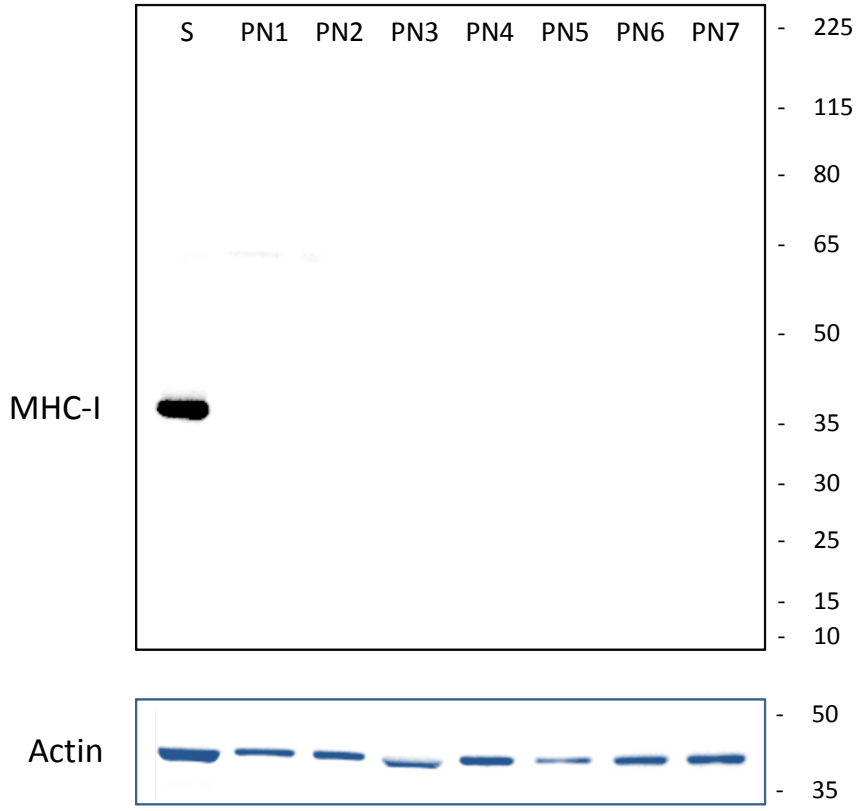


Figure 4.6 MHC-I expression in peripheral nerve. Western blot of proteins extracted from devil peripheral nerve samples (PN1-PN7) probed with a cocktail of MHC-I antibodies (clones TD5, TD26, TD35 and TD50). None of the samples (O/7) showed MHC-I expression. The protein from spleen tissue (S) used as a positive control shows the MHC-I band at 40kDa. Actin (blue bands) as loading control is showed under the main panel. Signal detection was achieved using a chemiluminescence substrate (Millipore) and images taken with a Chemi-Smart 5000 digital camera (Vilber Lourmat). Scale (molecular weight) at the right of the membranes is in kDa.

4.3.4 Analysis of MHC-I expression in devil tissues and DFTD tumours: Immunohistochemistry analyses

The western blot analyses showed in the previous section indicated that a range of primary DFTD tumours express variable levels of MHC-I. Expression ranged from no detectable expression to levels similar to that of the normal control tissues such as spleen or leukocytes. Primary DFTD tumours normally grow within the dermis as aggregated nests or cords surrounded by connective tissue. Angiogenesis is also common. Consequently, protein preparations from samples of primary tumours that contain only tumour cells were difficult to obtain. Immunohistochemistry of tissue sections was used to investigate if the variability in the levels of MHC-I expression in DFTD observed by western blot analysis was related to the heterogeneous cell composition of the tumour sample or truly reflected the altered expression in the cancer cells.

Tissue sections of 28 primary tumours and 13 metastases were labelled with the anti-devil MHC-I antibodies cocktail. Observation of the labelling of normal tissue surrounding the tumours was used to compare the levels of expression between different types of cells. Strong MHC-I labelling was observed in epithelial cells within the skin. This labelling was both cytoplasmic and associated with the cell nucleus, and was observed in virtually all the skin cells (Figure 4.7). Fibroblasts in the underlying dermis and those in the connective tissue surrounding the tumours also showed cytoplasmic labelling (Figure 4.7). In some samples, the epithelial cells showed clear MHC-I labelling associated with the cell membrane (Figure 4.8). Positive cytoplasmic expression was also observed in endothelial cells (Figure 4.8) and lymphoid cells in the spleen and lymph nodes.

In general, the intensity of the labelling in primary DFTD tumour cells was lower than that of cells of surrounding normal tissue. In tumours forming dense nodules, the cancer cells at the periphery of the tumour nest and close to the surrounding normal/connective tissue showed stronger labelling compared to that of the cancer cells towards the centre of the nest (Figure 4.7). In a few tumour samples some of the tumour cells showed strong MHC-I labelling. Regardless of the intensity, labelling in tumour cells in all samples was primarily associated with the cell nucleus and in some cases cytoplasmic.

Significantly, no MHC-I labelling appeared to be associated with the cell membrane of the tumour cells (Figure 4.8).

Similarly, negative to moderate MHC-I labelling was observed in the tumour cells of the DFTD metastases. Immunoreactivity was mainly peri-nuclear or cytoplasmic and never associated with the membrane (Figure 4.9).

A semi-quantitative assessment of the MHC-I immunolabelling indicated that 86% of the primary tumour samples (24/28) showed negative to weak labelling in more than 50% of the tumour cells. In 11% of the samples (3/28) there was moderate MHC-I labelling, while only one sample labelled strongly in more than 50% of the tumour cells.

Likewise, 85% of the DFTD metastases (11/13) were negative or labelled weakly in more than 50% of the tumour cells. Labelling of the rest of the samples (2/13) was moderate. Strong labelling was not observed in DFTD metastases.

Finally, tissue sections of peripheral nerves within the skin were analysed for the expression of MHC-I. The immunohistochemistry studies supported the western blot analyses. Schwann cells within peripheral nerve bundles showed negative MHC-I labelling compared to that of the supporting cells within the bundle or the surrounding stromal tissue (Figure 4.10).

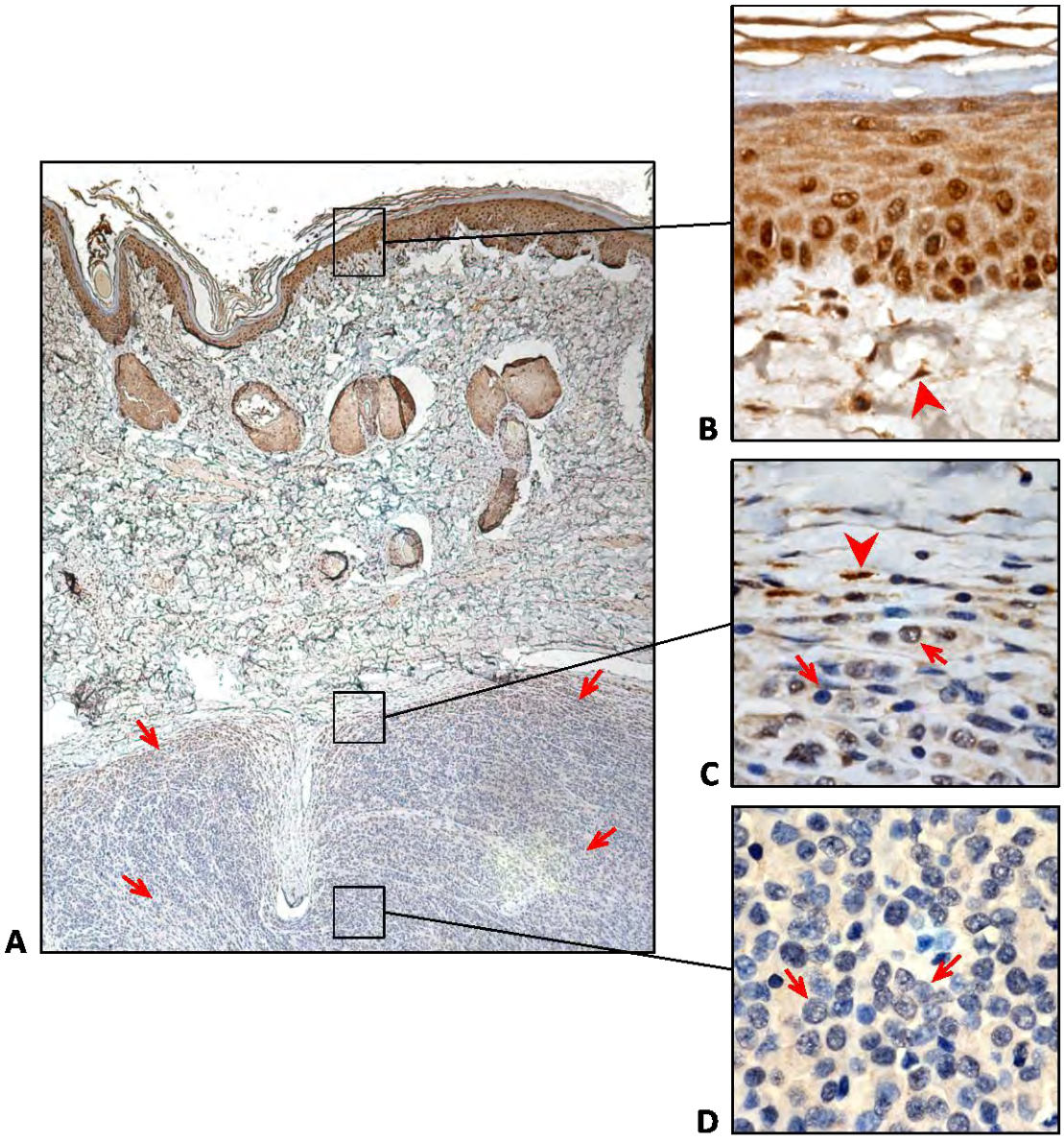


Figure 4.7 MHC-I expression in devil skin and DFTD tumour cells. **A**, Immunohistochemistry labelling of a section of devil skin containing a DFTD tumour nest (arrows) using a cocktail of anti-devil MHC-I antibodies (clones TD5, TD26, TD35 and TD50). DFTD cells show lower MHC-I expression compared to that of epithelial cells in the epidermis and normal cells within the dermis. Right panels: Magnification of the areas indicated with boxes in the left panel. **B,** Epithelial cells showing strong cytoplasmic and nuclear MHC-I labelling. Fibroblasts (arrowheads) and other cells in the dermis also show strong cytoplasmic labelling. **C**, Magnification of the area of contact between tumour cells (tumour nest periphery) and the surrounding stromal tissue. Tumour cells (arrows) show weak to moderate MHC-I labelling compared to that of the surrounding fibroblasts (arrowheads). **D**, DFTD tumours cells at the centre of the tumour nest showing negative MHC-I expression. Immunohistochemical detection with EnVision+ system (Dako) with hematoxylin counterstain.

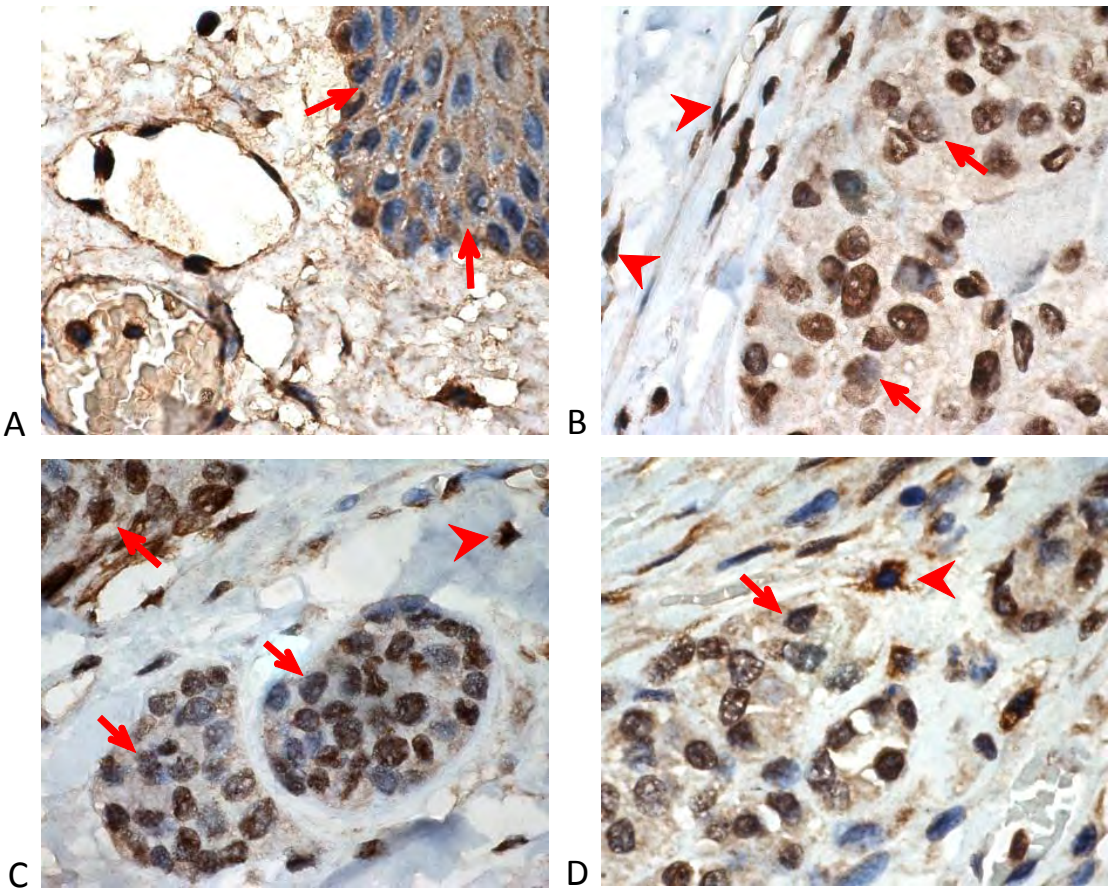


Figure 4.8 Patterns of MHC-I expression in normal devil tissues and devil facial tumours. **A**, Immunohistochemistry labelling of a section of normal epidermal tissues. Epithelial cells (arrows) show clear cytoplasmic MHC-I labelling associated with the cell membrane. Fibroblasts, endothelial cells, and leukocytes cells within blood vessel also show MHC-I labelling. **B**, DFTD tumour cells (arrows) show MHC-I labelling associated with the nucleus. Labelling intensity is similar to that of the surrounding normal cells (arrowheads). **C-D,** Variable MHC-I labelling of DFTD tumour cells (arrows). Normal cells (arrowheads) showing cytoplasmic MHC-I labelling. Immunohistochemical detection with EnVision+ system (Dako) with hematoxylin counterstain.

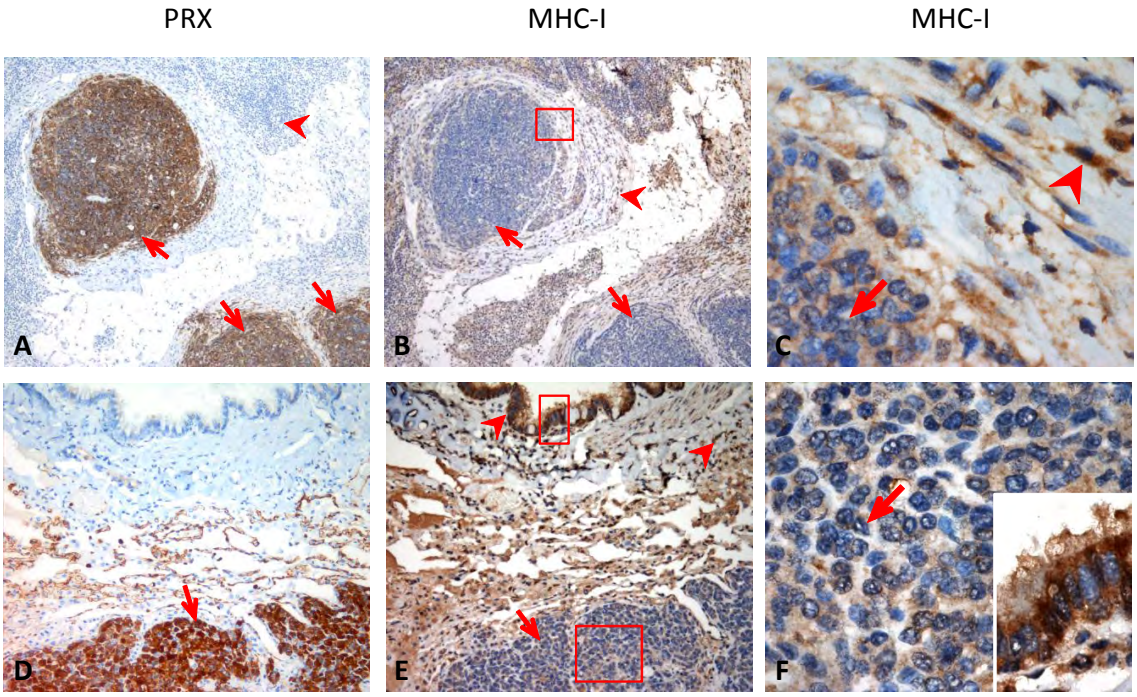


Figure 4.9 MHC-I expression in DFTD metastases. **A-C**, DFTD metastases in lymph nodes. **A**, Immunohistochemical labelling with periaxin antibody allows the detection of the tumour nests (arrows) within the normal lymph tissue (arrowhead). **B**, Same tissue section immunolabelled with MHC-I antibody. Normal connective and lymphoid tissue (arrowhead) shows MHC-I expression. DFTD metastases (arrows) show negative to very weak MHC-I labelling. **C**, Magnification of the area indicated by the box in the previous panel. Normal cells (arrowhead) show high intensity MHC-I labelling. DFTD cells (arrow) show weak cytoplasmic MHC-I expression. **D-F**, DFTD metastases in the lungs. **D.** Periaxin labelling that allows the detection of the tumour nests (arrows) within the normal lung tissue. **E**, DFTD cells (arrow) show very weak MHC-I labelling compared to that of the normal surrounding lung tissue (arrowheads). **F**, Magnification of the area indicated by the bottom box in the previous panel. DFTD tumour cells show weak cytoplasmic MHC-I labelling. The insert corresponds to the top box in panel E. Epithelial cells in a bronchiole show strong cytoplasmic MHC-I labelling that extends to the borders of the cell membrane. Immunohistochemical detection with EnVision+ system (Dako) with hematoxylin counterstain.

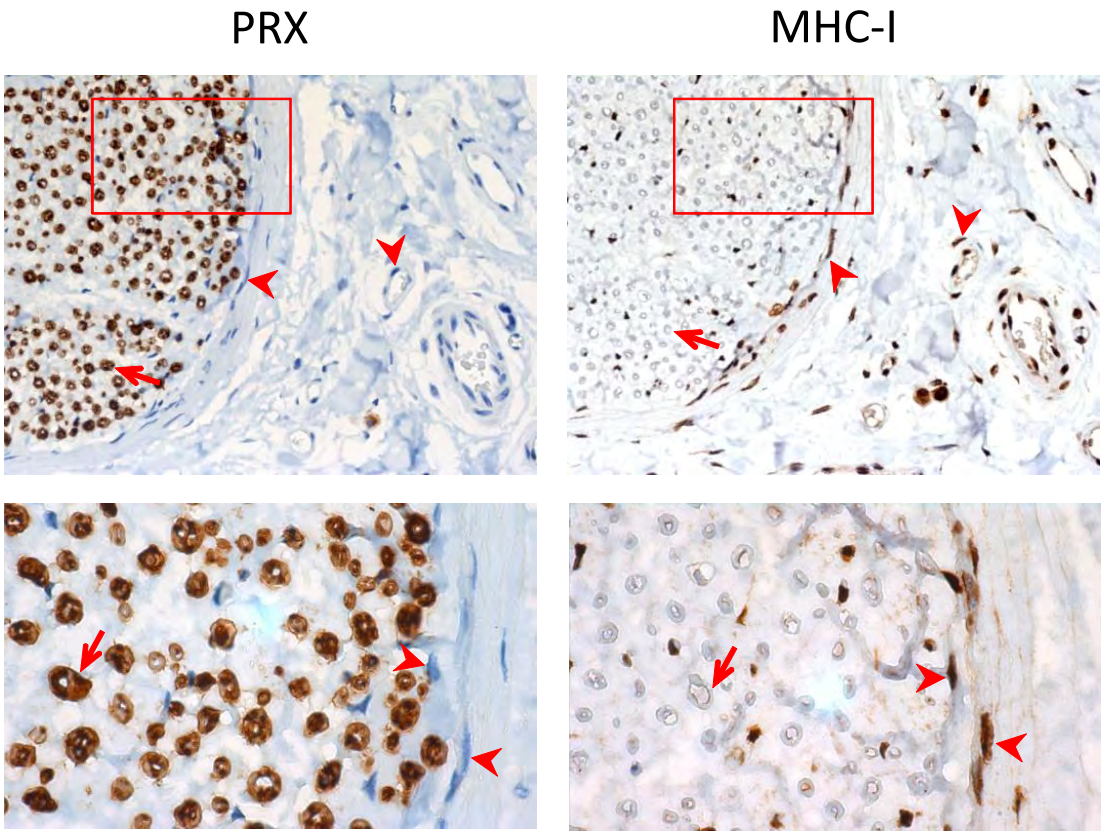


Figure 4.10 MHC-I expression in devil peripheral nerve. Left panels show immunohistochemistry labelling of a peripheral nerve bundle with periaxin antibody. Schwann cells within the bundle (arrow) show strong and specific periaxin labelling which is not observed in the surrounding tissue (arrowheads). The bottom left panel is a magnification of the area indicated in the top panel. Schwann cells (arrow) but not supporting cells show periaxin labelling. Right panels: the same tissue section labelled with a cocktail of MHC-I antibodies (TD5, TD26, TD35 and TD50). Stromal cells (arrowheads) show strong MHC-I immunolabelling. In contrast, most Schwann cells within the peripheral nerve bundle show no MHC-I expression. The bottom right panel is a magnification of the area in the box. Schwann cells within the bundle (arrow) express very weak levels of MHC-I protein. Supporting connective tissue cells within and surrounding the nerve bundle (arrowheads) show strong MHC-I labelling. Immunohistochemical detection with EnVision+ system (Dako) with hematoxylin counterstain.

4.3.5 Analysis of MHC-I expression in cultured devil fibroblasts and devil facial tumour cells: Immunofluorescence studies

Cells from devil ovary, uterus tissue and needle aspiration biopsies taken from devil facial primary tumours were plated onto coverslips and cultured for 8 days for immunohistochemical visualisation of MHC-I expression using confocal microscopy.

The primary cultured fibroblasts from devil ovary and uterus showed diffuse and punctate MHC-I labelling throughout the whole cytoplasm. MHC-I was colocalized with actin in the borders of the cells suggesting membrane expression (Figure 4.11).

In the cultures of primary devil facial tumours MHC-I labelling was detected in both fibroblast and tumour cells. However, the subcellular localization was different. Fibroblasts showed diffuse MHC-I labelling that colocalized with actin in the borders of the cells. In contrast, MHC-I did not colocalized with actin in the membrane extensions of the DFTD cells and labelling was mainly focused around the cell nucleus (Figure 4.12).

MHC-I labelling of tumour cell lines was restricted to the cell nucleus and peri-nuclear regions, similar to the labelling observed in the primary cultured tumour cells. There was no evident localization of the labelling at the outer borders or extensions of the DFTD cells (Figure 4.13).

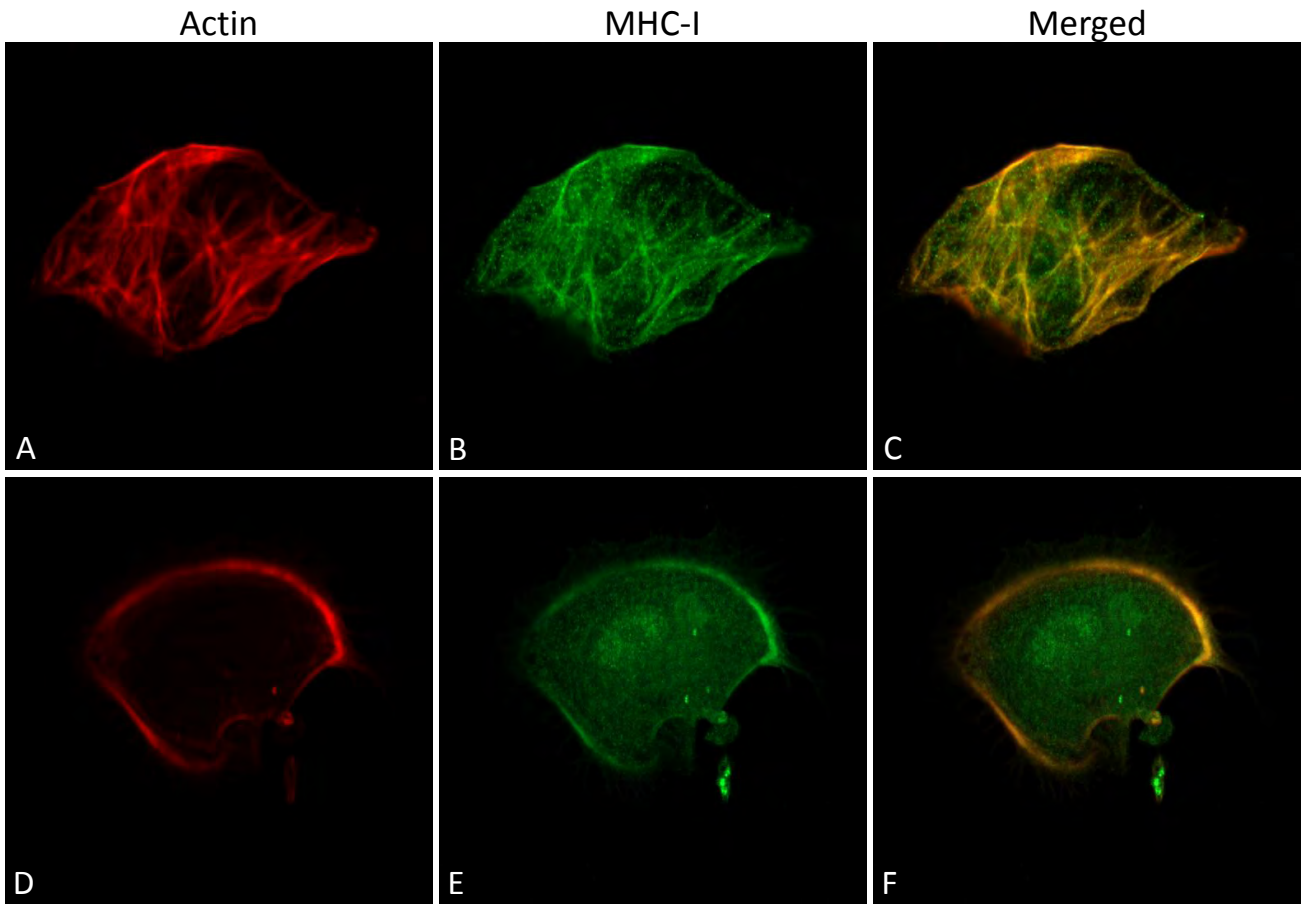


Figure 4.11 Cellular MHC-I localization in cultured devil fibroblasts. Confocal immunofluorescent microscopy analysis of MHC-I in primary cultures of devil fibroblasts extracted from ovary tissue (**A-C**) and fibroblasts extracted from devil uterus (**D-F**). **A**,**D**, Actin (red) staining. **B**,**E,** MHC-I labelling (green) is punctate and extends through the whole cell. **C,F**, Actin and MHC-I images merged. MHC-I colocalizes with actin at the periphery of the cell.

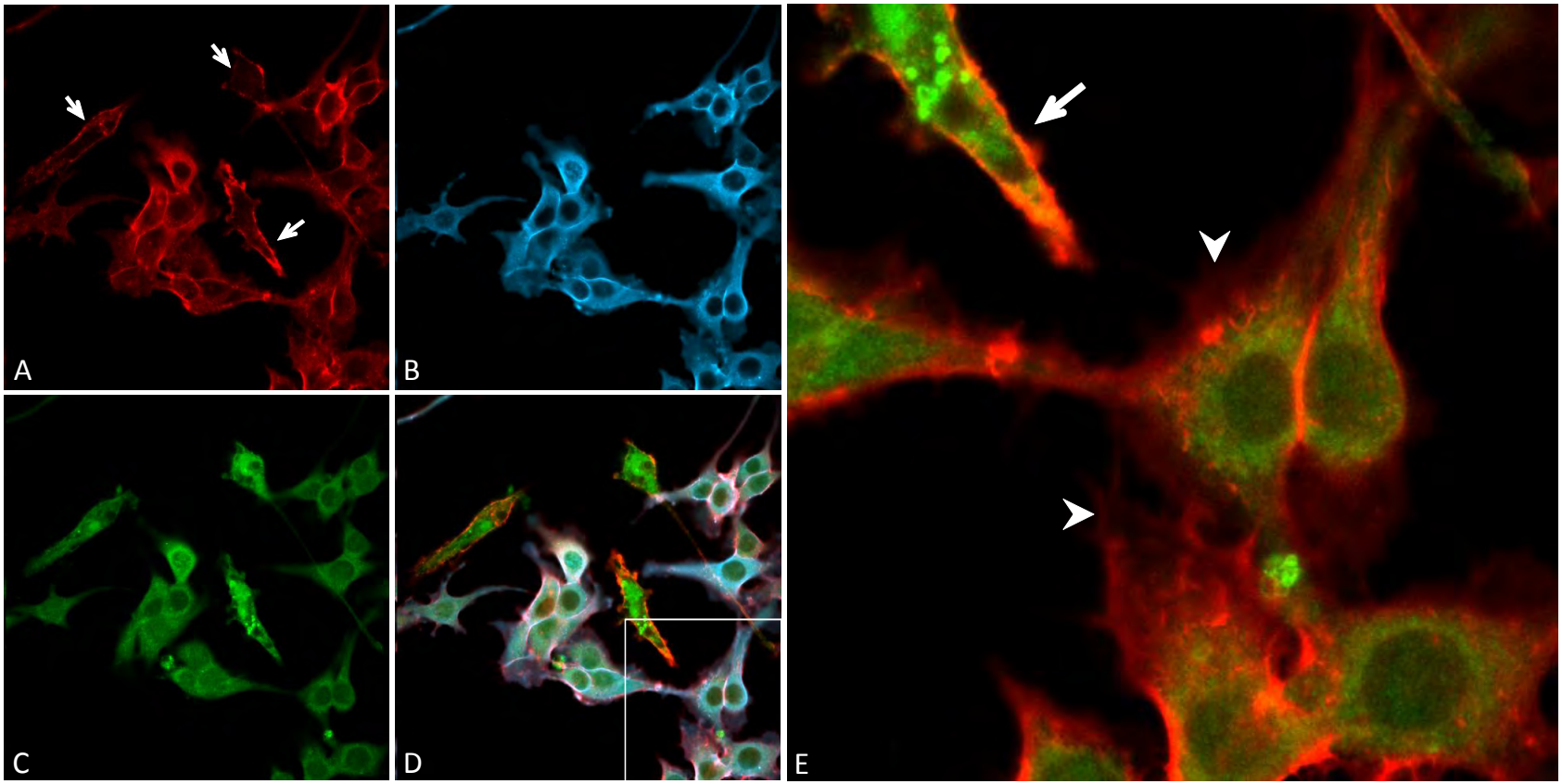


Figure 4.12 MHC-I colocalizes with actin in cell membrane extensions of fibroblasts but not in DFTD cells. Confocal immunofluorescent microscopy analysis of the cellular localization of MHC-I in primary cultured DFTD cells. Cells from needle aspiration biopsies from primary DFTD tumours were plated onto coverslips and immunolabelled with a cocktail of anti-devil MHC-I antibodies (TD5, TD26, TD35 and TD50), double labelled with periaxin and counterstained with actin (phalloidin). **A**, Actin staining (red) of DFTD cells and contaminating fibroblasts (arrows). **B**, Periaxin (cyan) only labels DFTD cells allowing them to be differentiated from fibroblasts. **C**, MHC-I labelling (green) is detected in fibroblast and DFTD cells. **D**, Images A, B and C merged. In DFTD cells MHC-I colocalizes with periaxin and actin mainly in the perinuclear area. **E**, Detail of the area indicated by the box in panel D. Actin (red) and MHC-I (green) are shown. In fibroblasts (arrows) MHC-I diffuses through the cytoplasm and colocalizes with actin at the membrane extensions of the cell. In DFTD cells (arrowheads) MHC-I labelling is perinuclear and does not extend to the cell membrane.

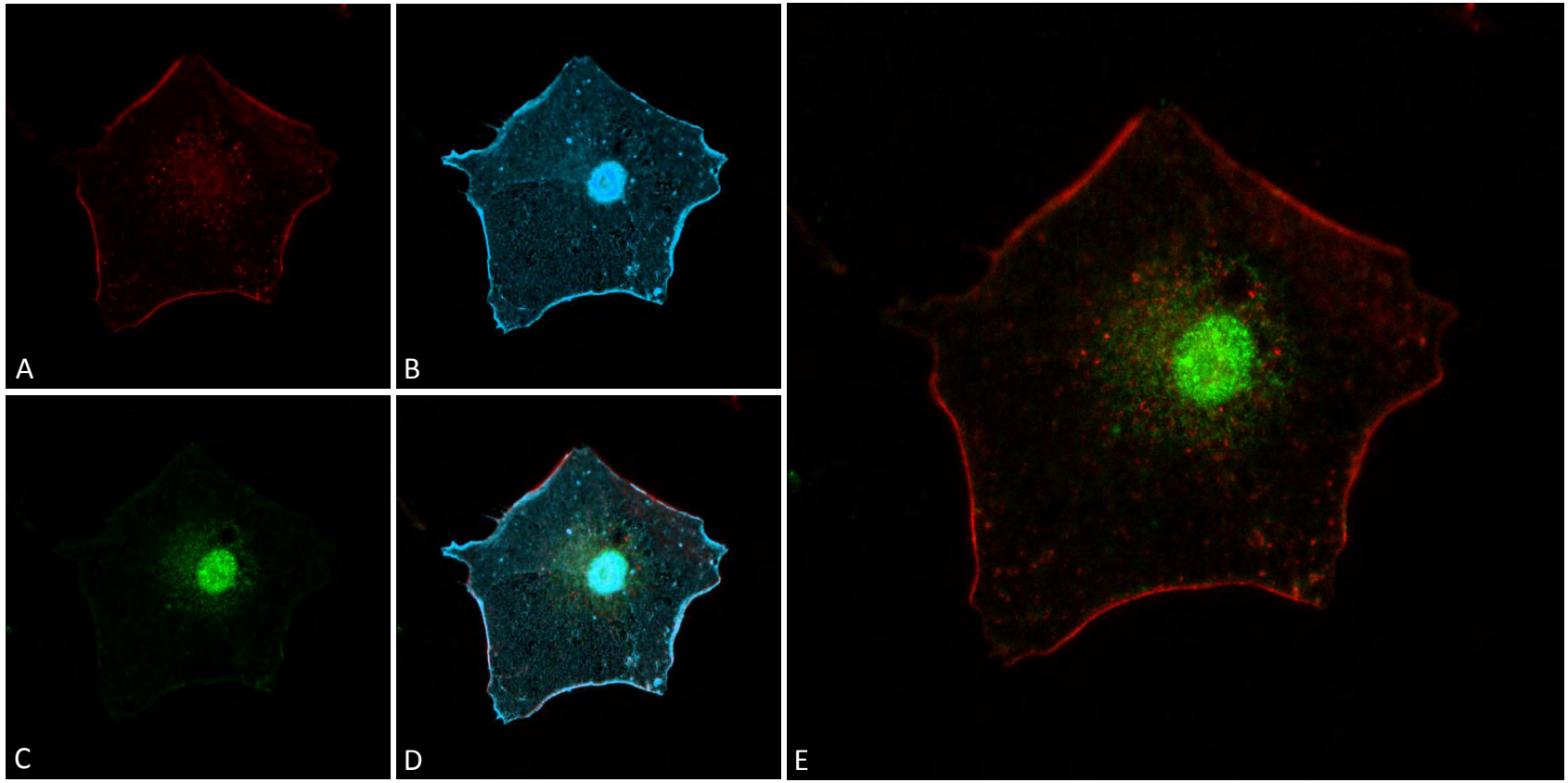


Figure 4.13 Cellular MHC-I localization in DFTD cell lines. Representative detail of the MHC-I labelling pattern in a DFTD cell using confocal immunofluorescent microscopy. **A**, Actin (red) staining. **B**, Periaxin (cyan) labels the cell through the whole cell surface. **C**, MHC-I labelling (green) is associated with the nucleus and the perinuclear region. **D**, Images A, B and C merged. **E**, Magnification of the cell showing only the actin (red) and MHC-I labelling (green). MHC-I does not colocalize with actin at the cell membrane.

4.4 Discussion

The mechanisms that allow transplantation of devil facial tumour cells across major histocompatibility (MHC) barriers are not clearly understood. The absence of an immune response to tumour establishment and growth was initially attributed to the lack of genetic diversity at the MHC loci (Siddle *et al.*, 2007a). However, recent studies showed that despite the limited allelic diversity, devils are capable of rejecting skin grafts and producing strong mixed lymphocyte reactions between individuals from different regions in Tasmania (Kreiss *et al.*, 2011a). The tumour has also spread to devil populations that were predicted to be resistant due to a greater MHC diversity (Hamede *et al.*, 2012). These lines of evidence suggest that the low MHC diversity alone cannot satisfactorily explain the lack of an immunological rejection to transferred tumour cells. This Chapter presents an integrated approach for the analysis of MHC-I protein expression in normal devil tissues and DFTD. Flow cytometry, western blot analyses, immunohistochemistry and confocal microscopy techniques were used to evaluate MHC-I expression in a variety of normal tissues, including leukocytes isolated from peripheral blood, primary devil facial tumours, metastases, and cultured devil fibroblasts and tumour cells. The combined results indicate that tumour cells have low levels and inappropriate localisation of MHC-I expression compared to that of normal tissues, such as epithelial, connective, endothelial and lymphoid tissue. This evidence provides an opportunity to consider other mechanisms of immune evasion that allow for the successful allotransplantation of tumour cells between devils in wild populations.

Immune evasion by downregulation of MHC molecules is common in cancer (Aptsiauri *et al.*, 2007). This particular mechanism explains the transplantation ability of neoplastic cells in the canine transmissible venereal tumour (CTVT), the only other naturally occurring contagious cancer (Cohen *et al.*, 1984). However, a similar analysis of the expression of MHC proteins in devils was hindered by the lack of cross-reacting reagents. In this study, 24 different antibodies against MHC molecules were initially screened for specificity against the devil MHC-I. These included widely utilised antibodies such as the clones MHC-2G5, MHC-W6/32 and beta-2-microglobulin, and antibodies against MHC-I molecules of the grey-short tailed opossum, a South American marsupial. Unfortunately, none of the antibodies were able to detect devil

MHC-I proteins. Recently, collaborators from the University of Cambridge (UK) developed the first anti-devil MHC-I antibodies raised against a synthetic peptide (GGKGGDYVPAAGN) representing the cytoplasmic tail of a known published devil MHC-I sequence (Siddle *et al.*, 2007b). These antibodies were used in this thesis to compare the expression of MHC-I in normal devil tissues and facial tumours.

The specificity of the anti-devil MHC-I antibodies was validated in this thesis by detection of a unique band of approximately 40 kDa (expected mass for MHC-I proteins) by western analysis of normal tissue proteins. Our collaborators in Cambridge also confirmed the specificity of the antibodies by western analysis of proteins isolated from cultured devil fibroblasts. In their experiments, the specific MHC-I band also disappeared after blocking with a synthetic MHC-I peptide (H. Siddle, personal communication, November 2011) further validating the specificity of the antibodies. However, as indicated previously, this thesis identified by western analysis an additional band (of approximately 60 kDa) present in most of the DFTD protein samples (i.e. primary tumours, cell lines and xenografted DFTD tumours). This band persisted in western analysis even following strong conditions of reduction and denaturation (data not shown). Furthermore, a band of similar molecular weight was also observed in the human cell line K562 (with the anti-devil antibodies) that lacks MHC-I. This band was not observed in normal devil tissue proteins. Therefore, it is unlikely that the detected protein is MHC related. It is possible that the antibody is binding non-specifically to an unusual protein characteristic of the cancer cell proteome. An attempt was made to separate the protein using SDS-gel electrophoresis and to identify the protein using mass spectrometry. Several proteins were identified within the predicted molecular weight but they were not MHC-I related (Appendix 4).

The additional band detected in the western blots should not affect the analysis derived from the immunohistochemistry studies. Four of the tumours samples were tested with both western blots and immunohistochemistry. One sample showed a weak band in the western blot analysis at both the correct (40 kDa) and additional 60 kDa bands. The other three samples only showed a weak band at 60 kDa, suggesting a lack of MHC-I expression. The immunohistochemistry for MHC-I was weakly positive, indicating that the non-specific binding was not detrimentally influencing these findings. As indicated

above, the extra 60 kDa band was only observed in tumour samples and not in normal tissues. At most, this low level of background in the immunohistochemistry of tumours might contribute to the "weak expression". This would be an overestimate of the actual labelling and further support the concept that MHC-I is downregulated. It is highly unlikely that it would account entirely for the stronger staining patterns.

MHC-I expression was first evaluated in normal devil tissues. Western blot analyses confirmed that MHC-I proteins are strongly expressed in kidney, spleen and leukocytes isolated from peripheral blood. Following this, the pattern of expression of MHC-I *in situ* was evaluated using immunohistochemistry. Strong MHC-I expression was observed in most cells within the skin including epidermal cells, fibroblasts and endothelial cells. Immune cells in lymph nodes expressed high levels of MHC-I protein. MHC-I expression was generally detected in the peri-nuclear region and diffusing throughout the cytoplasm. Clear MHC-I labelling associated with the cell membrane was observed in leukocytes, epithelial cells of the skin and epithelial cells in bronchioles. These combined results confirmed the synthesis of MHC-I proteins by normal devil cells. Detection of MHC-I associated with the cell membrane suggests that the underlying mechanisms and cellular components that regulate MHC-I translation, assembly and transport to the cell surface, are functional in Tasmanian devils.

The results from the western blot analyses indicated that MHC-I protein expression in tumours is vastly different to that of normal devil tissues. MHC-I protein was only detected in half of the protein samples from DFTD primary tumours. Although some tumours showed high levels of MHC-I protein expression, it is possible that this was due to contaminating stromal cells (e.g. connective tissue and blood cells) in a heterogeneous tumour sample.

In order to validate the western blot analyses, immunohistochemistry was used to study the *in situ* MHC-I expression in tumour sections. MHC-I protein was detected in DFTD cells of primary tumours. However, the levels of expression were always lower than that observed in normal cells such as epithelial cells, fibroblasts and leukocytes. Also the pattern of MHC-I expression in DFTD cells was atypical with most expression primarily perinuclear and in some cases cytoplasmic, but it was never observed to be associated with the cell membrane. These findings indicate that MHC-I protein

synthesis does occur in devil facial tumour cells. However, the immunohistochemistry suggests that these proteins are not transported to the cell surface.

Confocal microscopy was then used to increase the resolution and visualization of MHC-I expression patterns in normal and cultured DFTD cells. Cultured normal devil fibroblasts showed diffuse cytoplasmic MHC-I expression that also colocalized with actin at the cell membrane. MHC-I was also detected in primary and long term cultures of DFTD cells. However, as observed with immunohistochemistry, the expression in tumour cells was restricted to the perinuclear region. These results confirmed that although MHC-I is produced in DFTD cells, the molecules are not transported to the cell surface.

Thus, it is unlikely that MHC-I molecules in DFTD are immunologically functional. This relates to the capacity of MHC-I molecules of binding peptide fragments of intracellular proteins (antigens) and displaying them at the cell surface for antigen presentation to $CD8^+$ T cells (Donaldson and Williams, 2009). Low levels or lack of membrane MHC-I expression reduces cancer cell immunogenicity and would confer a survival advantage for DFTD cells. Low MHC-I surface expression would explain why devil facial tumour cells establish and grow undetected by the devil's immune system.

The cytoplasmic and perinuclear localization of MHC-I in devil facial tumour cells strongly suggest that MHC-I molecules are retained within the ER compartment and not exported to the cell surface. The antigen presenting machinery (APM) plays a crucial role in mediating immune responses by the generation and expression of the trimeric MHC-I, beta-2-microglobulin (B2M) and peptide complex (Seliger, 2012). Components of the APM include the catalytic beta subunit of the proteasome, the transporter associated with antigen processing (TAP-1 and TAP-2) and various endoplasmic reticulum (ER) chaperones (Donaldson and Williams, 2009). Therefore, it is probable that deregulation of APM components is also occurring in DFTD.

Under normal circumstances, the levels of MHC-I and APM components expression are transcriptionally and epigenetically regulated. Different cells and tissues can constitutively express widely different levels of MHC molecules. For instance, MHC expression is highest in the cells of the immune system whereas MHC is usually

undetectable in the cells of the central nervous system and myocardial cells (Hutchinson, 2010). Furthermore, MHC transcription in a given tissue can change in response to hormonal or cytokine stimuli (Lee *et al.*, 2010). This Chapter showed that normal devil tissues, such as epithelial cells within the skin and lung, and cells of immune organs, express high levels of MHC-I. On the contrary, Schwann cells within peripheral nerves bundles showed low or no MHC-I expression. MHC-I was not detected in samples of protein extracted from adult peripheral nerves. The fact that DFTD cells and Schwann cells showed low levels of MHC expression strongly suggests that the trait relates to the cell of origin rather than acquired after cell transformation.

In normal cells the epigenetic and transcriptional modulation of MHC-I expression involves the existence of underlying regulatory mechanisms that control and modulate gene expression. Mechanisms include DNA methylation, post-translational modifications of histones, transcription factors, and microRNA patterns among others (Esteller, 2008, Seliger, 2012).

A differential expression pattern of MHC-I was observed in the different DFTD models investigated in this thesis suggesting the occurrence of these modulatory mechanisms. Evidence for this includes:

- Different levels of MHC-I expression among primary DFTD tumours from different animals.
- Different levels of expression within a single tumour with cells at periphery of the tumour nest showing more intense MHC-I immunolabelling.
- Lower MHC-I expression in DFTD metastases than primary tumours.
- Differential expression of MHC-I among cell lines, with protein detected only in one cell line.
- No MHC-I expression detected in the DFTD tumours xenografted onto mice.

Low levels of MHC-I expression in DFTD cells would reduce immune recognition. This is comparable to the frequent strategy employed by tumours to evade immune detection. This is, the deregulation of these modulatory mechanisms, which in turn alter MHC-I expression (Cabrera *et al.*, 2003). MHC-I deficiencies have been described in human cancers such as cervical carcinoma, colorectal carcinomas, breast carcinoma, melanoma and bladder carcinoma (Aptsiauri *et al.*, 2007, Ferrone and Marincola, 1995, Koopman *et al.*, 2000) and the canine transmissible venereal tumour (CTVT) (Cohen *et al.*, 1984). Various mechanisms are responsible for these alterations and involve structural changes (genetic mutations or deletions), deregulation of the expression of components of the antigen presenting machinery (APM) or epigenetic changes in the DNA (Seliger *et al.*, 2002). What are the leading mechanisms that control MHC-I expression in DFTD cells is an area that requires further investigation.

In summary, this chapter showed that MHC-I proteins are expressed in DFTD tumour although at lower levels than normal tissues. More importantly, MHC-I is not expressed at the cell surface suggesting that impaired expression of associated components of the antigen processing machinery is also involved. Alteration of MHC-I surface expression reduces the immunogenicity of DFTD cells and therefore plays a significant role in the lack of immune recognition to transferred DFTD cells. Thus, this information provides new insights into the mechanisms of immune evasion that allow allotransplantation of devil facial tumour cells. It also opens new areas of research for the development of management strategies for the conservation of the species such as immunotherapy and vaccination.

5 An immunoproteomic approach to identify devil facial tumour associated antigens

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5.1 Introduction

Results from the previous chapter indicated that downregulation of MHC-I proteins at the cell surface may impede immune recognition of devil facial tumour cells. These findings imply that DFTD cancer cells are unlikely to encounter natural immunohistocompatibility barriers. Despite the immune system of devils being able to recognise and reject allogeneic transplants (Kreiss *et al.*, 2011a), it would be unlikely to find animals that are resistant to DFTD in the wild. Vaccination would provide a potential strategy for the conservation of the species should a suitable antigen be identified.

Recent immunisations of devils with non-viable DFTD cells performed by our research group induced antibody responses. This type of humoral response against tumour cells is common. In humans, it is well established that antibodies against tumour antigens can occur in cancer patients (Reuschenbach *et al.*, 2009). The tumour antigens eliciting an immune response are collectively called tumour-associated antigens (TAAs).

Molecular studies revealed that TAAs are the consequence of the genetic and epigenetic alterations in cancer cells or the products of genes encoding viral proteins (Matsushita *et al.*, 2012, Pardoll, 2003, Schreiber *et al.*, 2011). The reason for spontaneous humoral responses in cancer are not known but may include new epitopes in expressed proteins, altered post-translational modifications with immunological relevance (e.g. underglycosylation), altered tissue specific expression patterns or over-abundance of the antigen (Fonseca and Dranoff, 2008, Houghton, 1994, Vesely *et al.*, 2011).

Different strategies are currently available for the identification of TAAs and more than one hundred have been described from human tumours. The detection of antibodies against TAAs in cancer patients has been a useful tool for early cancer diagnosis, prognosis and targeted immune therapy (Buonaguro *et al.*, 2011, Jager *et al.*, 2001). TAAs have also contributed to the understanding of the tumourigenesis process and cancer biology (Bortner *et al.*, 2009, Cappello *et al.*, 2003b, Ciocca and Calderwood, 2005).

The identification of the DFTD antigens eliciting humoral responses in Tasmanian devils may provide valuable information about this emerging disease and contribute to the development of a vaccine. Consequently, this chapter presents an immunoproteomic approach to investigate tumour-associated antigens in DFTD.

5.2 Methods

5.2.1 Experimental approach

Immunoproteomics, which describes the subset of proteins involved in the humoral response, has been a potential tool for the discovery of serological markers (Imafuku *et al.*, 2004). This section describes the development of an immunoproteomic approach for the identification of cognate DFTD antigens eliciting humoral responses in immunized Tasmanian devils. In this strategy, tumour proteins are first separated on replicate gels using two-dimensional gel electrophoresis (2DE), transferred to nitrocellulose membrane and probed with pre-immune devil sera, immune devil's or control antibodies. A replicate gel is also stained with coomassie blue. Immunoblots detected with sera from immunized animals are then compared to those of the controls (pre-immune sera), differentially expressed spots are then located on the coomassie-stained gel and excised. Excides proteins are subjected to trypsin digestion and finally identified by mass spectrometry (Figure 5.1)

5.2.2 Tasmanian devil immunisations

Experiments involving the use of Tasmanian devils were conducted under the approval of the University of Tasmania Animal Ethics Committee (permit number A0009215).

A DFTD cell line (C5065) was kindly provided by the Tasmanian Department of Primary Industries, Parks, Wildlife and Environment (DPIPWE). The cells were maintained in RPMI culture medium (RPMI 1640 – Gibco, New Zealand) containing 10% foetal calf serum, 2 mM of L-glutamine (Sigma, USA) and 40 mg/ml of gentamicin (Pfizer, Australia) at 35 °C in a humidified atmosphere of 5% CO_2. To generate cells for immunisations, DFTD cells were frozen in 1 ml aliquots at a concentration of 1×10^8 cells/ml in sterile PBS. On the day of immunisation cells were thawed and sonicated for four cycles of 60 seconds at input 6 (out power 6 watts), with 60 second intervals on ice using a Microson™ Ultrasonic Cell Disruptor (XL 2000, Qsonica, Newtown, CT). The suspensions were centrifuged at 960 x g and the cell pellets were resuspended in 1 ml of sterile PBS. The cell suspension was mixed with 400 µl each of Montanide ISA 71 MVG adjuvant (Seppic, Puteaux, France) and CpG 1668 (450µg).

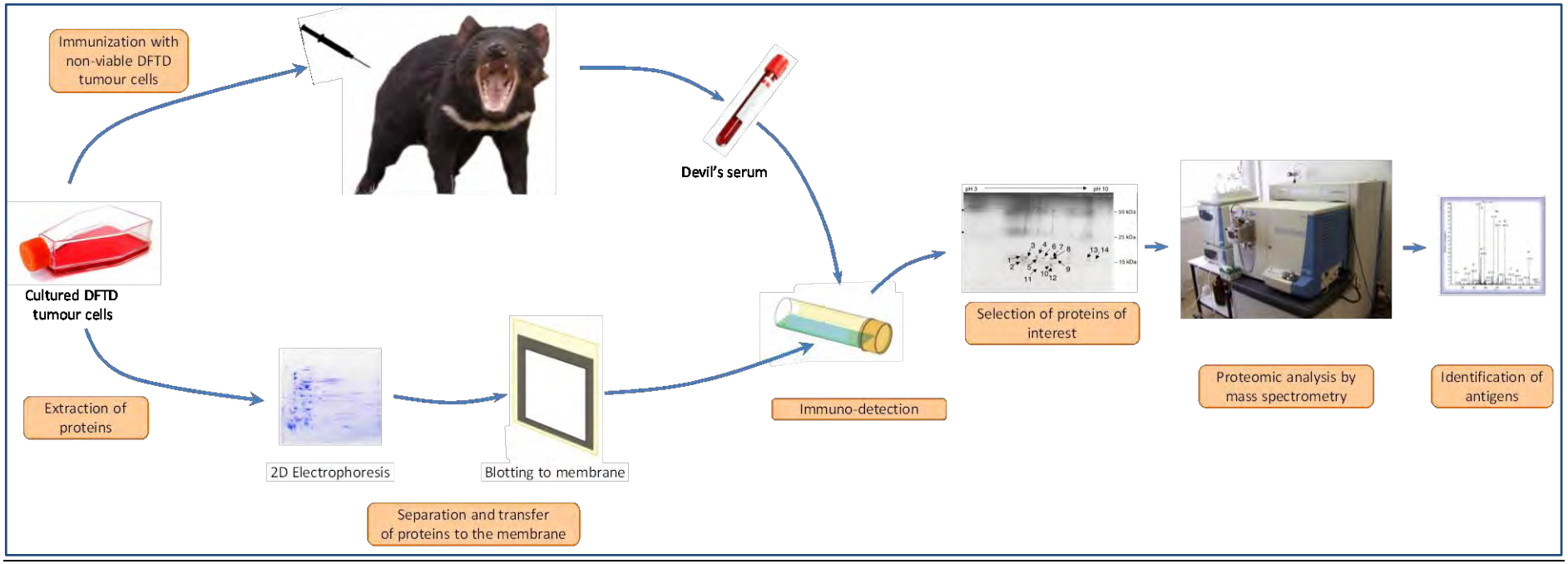

Figure 5.1 Schematic illustration of the immunoproteomic approach for identification of devil facial tumour associated antigens.

Two anaesthetised healthy male Tasmanian devils were injected sub-cutaneously in the shoulder with the cell suspensions at monthly intervals (three times) and blood samples collected every 14 days post- injection in clot-activating tubes (approximately 10 ml of blood was taken from the jugular from the anaesthetised devil as described in section 4.2.1). Approximately 8 ml of blood (from a 10 ml sample) was injected into a lithium-heparin tube for isolation of leukocytes and the remaining volume of the sample was used for extraction of serum. The blood samples were centrifuged at 1100 × *g* for 10 minutes and serum harvested. Clot removed and the process repeated. Serum was aliquoted into 200 µl samples and stored at -20 ^{o}C.

5.2.3 Preliminary screening of serum samples

An initial screening of the devil's serum from immunised and healthy animals was performed using standard denaturing one-dimensional gel electrophoresis (1D-SDS-PAGE). Optimization of the concentrations of antibodies for immune detection (i.e. devil serum, polyclonal rabbit anti-devil immunoglobulin and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG) was performed using the Mini-PROTEAN II Multiscreen apparatus (Bio-Rad, USA). This system allowed the simultaneous screening of multiple samples by western analyses using minimal amount of reagents. The conditions of electrophoresis, protein transfer and immunodetection (including details of antibodies) are explained below.

SDS-electrophoresis

Total DFTD proteins from cell lines were extracted with RIPA buffer (Thermo Scientific, Rockford, IL) as described in section 4.2.2. 400-500 µg of protein in loading sample buffer (3X) was heated at 95 ^{o}C for 10 minutes prior to loading on a single-well 7% polyacrylamide gel using the Xcell SureLock™ mini-cell (Invitrogen, Carlsbad, CA) and running buffer (see Appendix 2 for a list of reagents). The gels were run at 100 V until the line of the dye reached the bottom of the gel.

Protein transfer

Transfer of proteins from gels to a nitrocellulose membrane (0.45 µm, Bio-Rad, Germany) was performed using the XCell II™ Blot Module system (Invitrogen).

Blotting was performed using 1XNuPAGE® (Invitrogen) transfer buffer with 10% methanol overnight at 4 ^{o}C and 25 V.

Immunodetection

Immunodetection was performed using a Mini-PROTEAN II Multiscreen apparatus following the instructions of the manufacturer. Devil serum was applied at a dilution of 1/2000 in TBS buffer containing 0.1% tween 20 and 5% skim milk (TBSTM). After four washes with TBST, the secondary antibody (polyclonal rabbit anti-devil immunoglobulin) was applied at a dilution of 1/2000 in TBSTM. Production of this secondary antibody was previously described in Kreiss (2009). Briefly, rabbits were immunised with devil immunoglobulins that were previously purified by precipitation with ammonium sulphate from whole devil's serum. The rabbit IgG fraction was then purified using a protein A antibody purification kit.

Sera from naïve devils and the pre-immune sera from the immunised devils were used as controls. Two negative controls were run. One omitting the primary antibody (devil's serum) and another control omitting both primary and secondary antibodies. A commercial antibody against the cytoskeleton protein actin (beta actin polyclonal antibody ab729, Abcam) was used as loading controls for the western blot analyses.

The membrane was finally incubated with a donkey horseradish peroxidase (HRP)-conjugated anti-rabbit IgG tertiary antibody (NIF 824, Amersham Biosciences, Piscataway, NJ) at a dilution of 1/5000 in TBSTM. Chemiluminescent detection was performed following membrane immersion with Immobilon™ Western HRP substrate (Millipore, Billerica, MA) for 5 minutes at room temperature using a Chemi-Smart 5000 digital camera (Vilber Lourmat, EEC).

5.2.4 Two-dimensional electrophoresis (2DE)

2DE was performed using the ZOOM® IPGRunner™ System and the Xcell SureLock™ mini cell (Invitrogen, Carlsbad, USA) following the instructions of the manufacturer. A summary of the protocol is presented here. Complete instructions can be found in the manual of the system (ZOOM® IPGRunner™ System – Manual

MAN0000256 – Invitrogen, see Appendix 5). Appendix 2 includes a comprehensive list of reagents.

Sample preparation

A 950 µl aliquot of chilled lysis buffer containing ZOOM® 2D protein solubilizer, 1 M tris-base, protease inhibitor cocktail and 2 M DTT, were added to 50 µl of pelleted DFTD cells and the sample sonicated. The lysate was then alkylated with N,N-dimethylacrylamide (DMA) and excess DMA quenched with 2 M DTT, centrifuged, and the supernatant transferred to a new microcentrifuge tube.

A 12 µl aliquot of lysate containing ~15 µg of protein was diluted with sample rehydration buffer containing 1X ZOOM® 2D protein solubilizer, 2 M DTT, ZOOM® carrier ampholytes and bromophenol blue.

First dimension – isoelectric focusing (IEF)

155 µl of the protein in sample rehydration buffer was used to rehydrate each ZOOM® strip pH 3-10NL overnight using the ZOOM® IPGRunner™ cassettes.

After rehydration, isoelectric focusing was performed using the ZOOM® IPGRunner™ and the XCell SureLock™ mini-cell. Electrofocusing conditions were 200 V for 20 minutes, 450 V for 15 minutes, 750 V for 15 minutes and finally 2,000 V for 4 hours.

Second dimension SDS-PAGE

IEF strips were equilibrated with NuPAGE® LDS sample buffer and NuPAGE® sample reducing agent and then alkylated with alkylation solution containing NuPAGE® LDS sample buffer and iodoacetamide.

The equilibrated strips were then applied to second dimension gels (Nu NuPAGE® Novex® 4-12% Bis-Tris ZOOM® gels). SDS-PAGE gel-electrophoresis was performed at 200 V for 40-50 minutes using the NuPAGE® system and the XCell SureLock™ mini cell (Invitrogen, Carlsbad, CA).

Protein Transfer

Electroblotting of proteins from the gel to nitrocellulose membrane was performed using the iBlot® dry blotting system (Invitrogen). Blotting was performed using 20 V for 8 minutes.

Immunodetection

Immunodetection was performed as described in section 5.2.3 with the following antibody concentrations: Tasmanian devil serum (primary antibody) at 1/2000; secondary rabbit anti-devil immunoglobulin at 1/7500 and the HRP-conjugated anti-rabbit at 1/5000. Pre-immune sera from the immunised devils were used as a control. A negative control omitting the primary antibody (devil's serum) was also run.

5.2.5 Mass spectrometry analysis and selection of proteins

In-gel digestion

2DE gels were stained with GelCode™ Blue Safe Protein Stain (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Matched immunospots were identified in the gel, excised with a scalpel and transferred to microcentrifuge tubes. In-gel trypsin digestion was performed using the In-Gel Tryptic Digestion Kit (Thermo Scientific, Rockford, IL, USA).

Mass spectrometry and analysis of data

Digested samples were processed and analysed by the Central Science Laboratory at the University of Tasmania using multidimensional protein identification technology (MudPIT) and a tandem mass spectrometer (LTQ Orbitrap, Thermo Scientific, San Jose, CA).

Mass spectra data were searched against a database consisting of semi-tryptic digestion of marsupial proteins (Metatheria - NCBI) using X!Tandem v2007.07.01 software. Positive protein identification required two or more unique matching peptides (Kocharunchitt *et al.*, 2012). Where multiple proteins were reported (e.g. tubulin isoforms) peptides were assigned to satisfy the principal of parsimony.

5.3 Results

5.3.1 One-dimensional gel-electrophoresis

A preliminary screening using the sera of two devils that had been immunized with DFTD cells showed several unique bands that were not detected when using pre-immune sera. The pattern of bands using the pre-immune sera was similar to that of the sera from four naïve devils (D1-D4). However, the resolution and visualization of the bands, particularly in the lower molecular weight range, was not very clear due to use 7% polyacrylamide gels for protein separation and immune detection through a mini-multi screen apparatus (Figure 5.2). The system proved useful for both screening multiple samples and optimization of antibody concentrations.

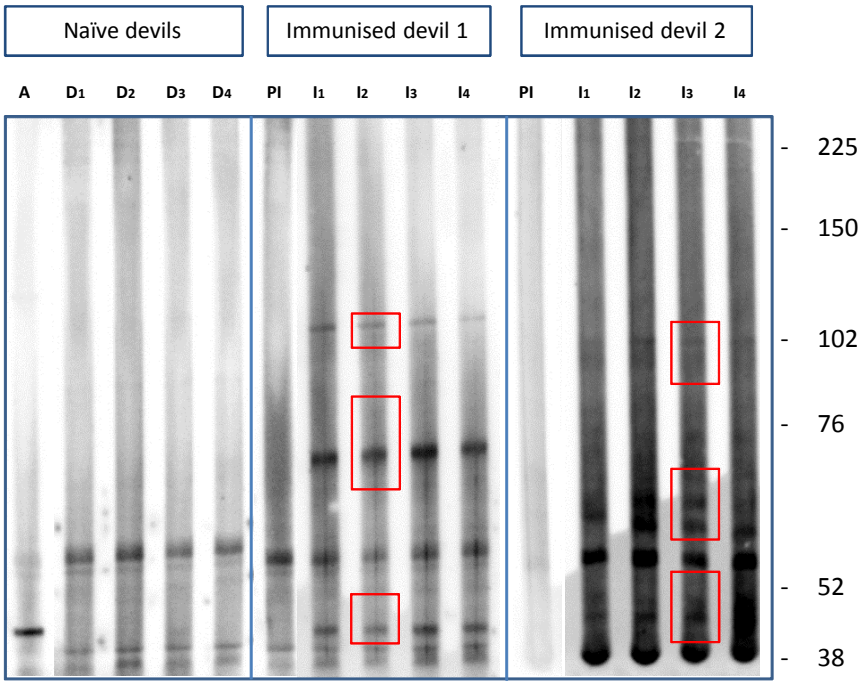

Figure 5.2 Screening of Tasmanian devils' serum for detection of antibodies against DFTD proteins using western blot analysis. Total DFTD proteins from cells in culture were separated by regular one-dimensional gel-electrophoresis (7% polyacrylamide) and blotted to nitrocellulose membrane. Membranes were probed with sera from four naïve devils (D_1-D_4) or sera (I_1-I_4) from two immunised devils. Pre-immune sera samples (PI) for each devil were run in parallel. Devil immunoglobulins were detected using polyclonal rabbit anti-devil and HRP-conjugated anti-rabbit IgG antibodies. Chemiluminescence was used for signal detection. Red squares indicate unique bands not detected in the sera of naïve devils or the pre-immune sera. Lane A, actin control

To increase resolution, DFTD proteins were separated using precast gradient gels (4-12%). This system facilitated the detection of clear bands in the serum of the two immunized devils. A stronger signal and better resolution of bands was observed when

using the serum from devil 1, as compared to devil 2. Higher levels of background were also persistently observed using serum from devil 2 compared to the background observed with serum from devil 1.

This analysis identified three major findings. Firstly, the sera from normal devils (non-immunised) contained antibodies that reacted against DFTD proteins, as expected. The use of pre-immune sera resulted in the identification of four DFTD-specific protein species within the 35-70 kDa range. Secondly, a number or unique bands were detected using the sera of immunised devils. Two new bands around 100 and 70 kDa were clearly observed plus several bands within the 10-35 kDa range. Third, the polyclonal rabbit antibody used to detect the devil's immunoglobulins, when used alone (i.e. without devil serum) produced a strong background band around 80 kDa and one less evident background band at 60 kDa (Figure 5.3).

5.3.2 Two-dimensional electrophoresis (2DE)

The previous results showed that the immunisation of devils with DFTD cells produced an immune response that was detected using western blot analysis. Thus, the next step was to pursue identification of the DFTD antigens responsible. For this, the DFTD proteins were separated by 2D-gel electrophoresis and antigen-antibody reactions detected using western blot analysis as described above. Spots of interest were excised from the gel and, after in-gel digestion, analysed by mass spectrometry.

Approximately 15 unique spots were detected using serum from immunised devil 1, and 5 unique spots using sera from immunised devil 2. At least one of the spots, around 15 kDa, was common to both samples. The pre-immune serum from both devils showed some spots that were clearly different from any spots observed using sera from immunised devils. The rabbit polyclonal anti-devil antibody again produced two background bands of about 60 and 85 kDa (Figure 5.4).

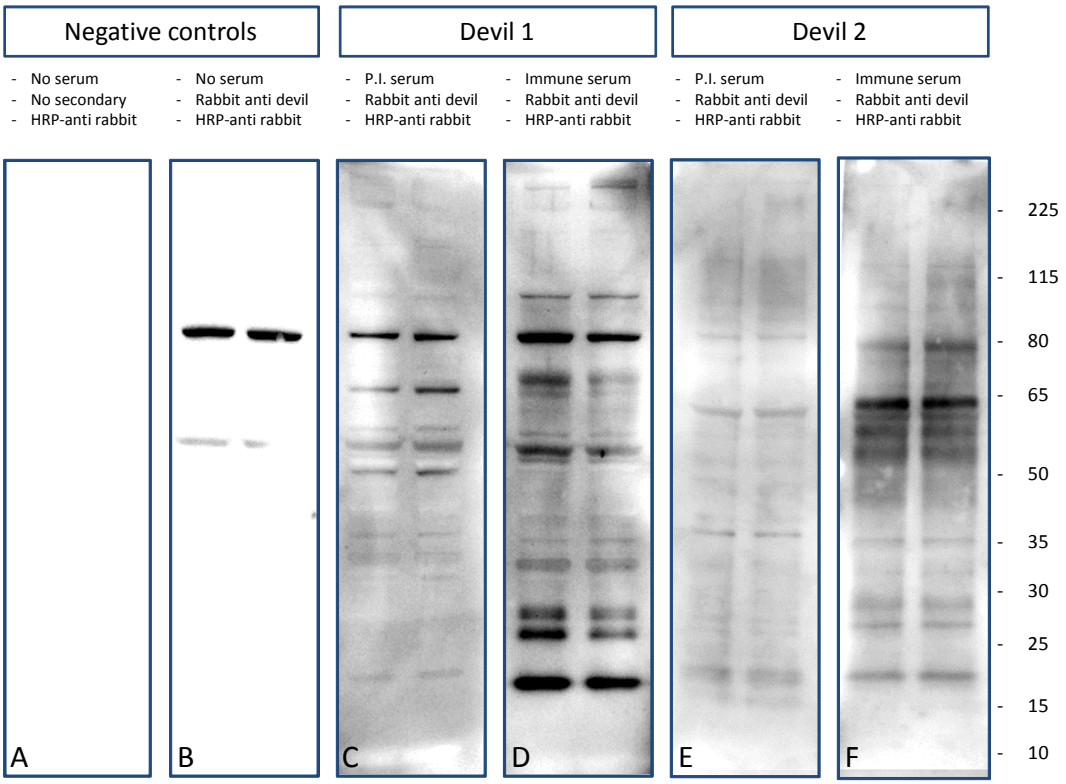

Figure 5.3 Western blots of DFTD cell protein separated by one-dimensional electrophoresis and probed with pre-immune (P.I.) and immunised sera from Tasmanian devils. Two replicates (lanes) were run for each sample. A range of bands were observed with the immune sera of both devils (**D** and **F**), which were not detected using pre-immune (P.I.) sera (**C** and **E**). Negative controls are also shown (**A** and **B**). Two background bands (~80 and 60 kDa) were detected using the secondary polyclonal anti-devil immunoglobulin (**B**).

Based on these results, a preliminary experiment was carried out in order to identify and characterize the antigens detected using the serum of immunised devil 1. To do this, matching spots from the immunoblots were identified in duplicate coomassie stained 2DE gels. In some instances, a single immunospot covered more than one single protein in the gel. Thus, extra gel spots were taken (Figure 5.5). Twenty spots were finally excised from the 2DE gel and following trypsin digestion, analysed by MudPIT. This resulted in the identification of 29 different proteins (based on two or more unique peptides detected). In some cases, several isoforms for a single protein were detected. In this case, all isoforms were collectively counted as one protein (Table 5.1).

The background present when the polyclonal anti-devil immunoglobulin antibody is used alone, was found to correspond to spots 19 and 2. Four different proteins were

characterized in spot 19, which were not identified in other spots. Four proteins were identified in spot 2. However, the same proteins were also identified in adjacent spots that are not related to the background band. Therefore, precaution should be taken when considering these proteins (unique proteins identified in spots 2 and 19) true tumour-associated antigens.

The protein from spot 3, identified as dachshund homolog 2, was immunodetected in both the pre-immune and immune sera from devil 2.

In summary, 24 unique antigens were identified using serum from immunised devil 2. One antigen, characterized as protein stathmin (spot 16), corresponded to the antigen recognised using sera from both immunised devils.

No proteins, meeting the criteria for inclusion (i.e. two or more unique peptides) were identified from spots 8 and 13.

5.3.3 Biological relevance of the identified proteins

A search of the literature using the PubMed database was performed in order to understand the biological relevance of proteins identified. Attention was given to proteins that have been previously identified as tumour antigens or as being associated with an immune response to cancer. From this search, 17 proteins were found to have well-documented information for their relation to cancer. Table 5.2 summarises these results.

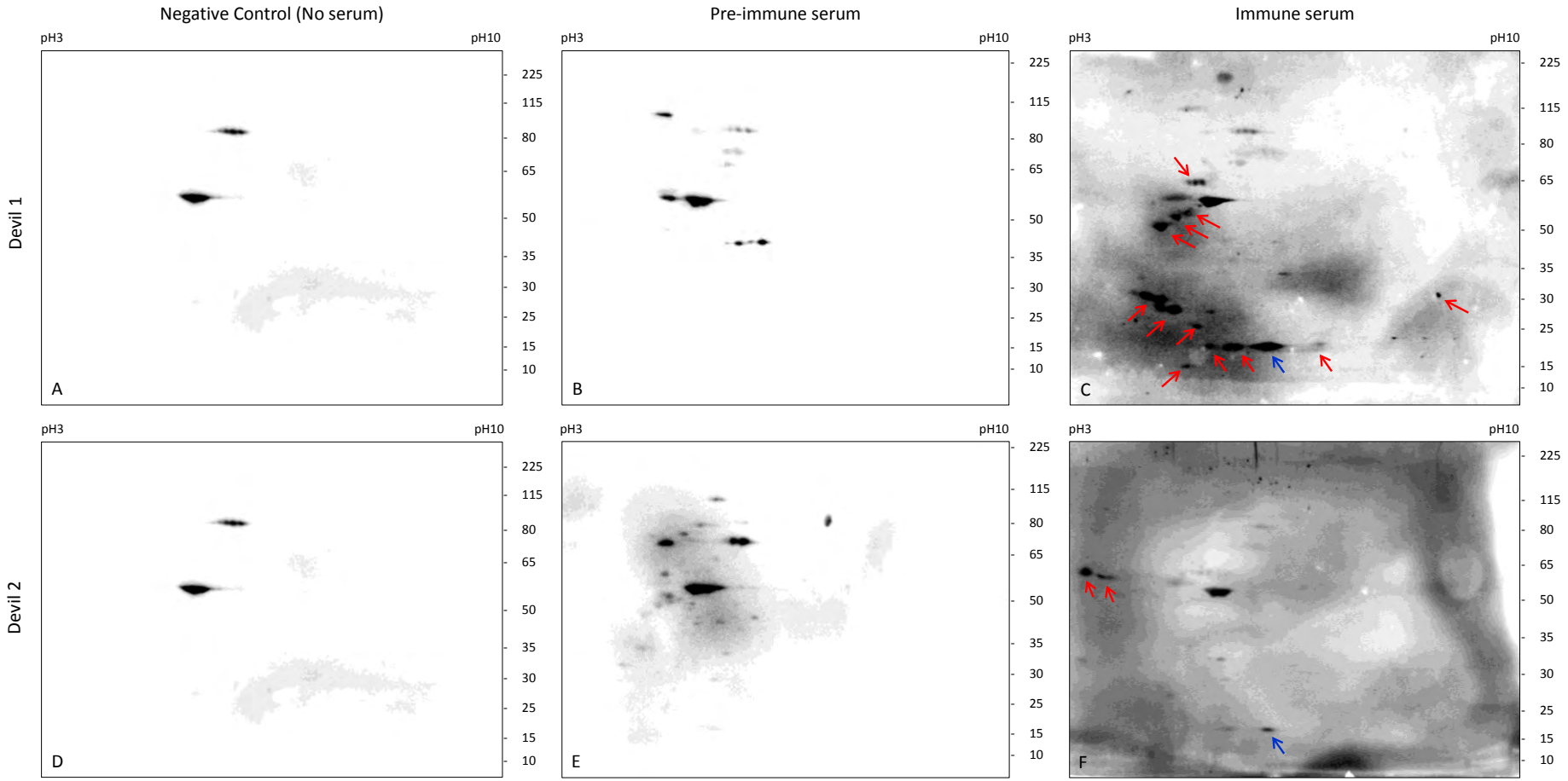

Figure 5.4 Screening of antibodies directed against DFTD tumour antigens using sera from immunised Tasmanian devils. Total cell proteins from cultured DFTD cells were separated by two-dimensional gel-electrophoresis, transferred to nitrocellulose membrane and probed with pre-immune sera (**B** and **E**) or post-immunisation sera (**C** and **F**). Negative controls (no serum) (**A** and **D**) are shown. Red arrows indicate spots recognised by sera of immunised devils, which were not detected using pre-immune sera or in negative controls. The blue arrow indicates a spot recognised by the immunised sera in both devils.

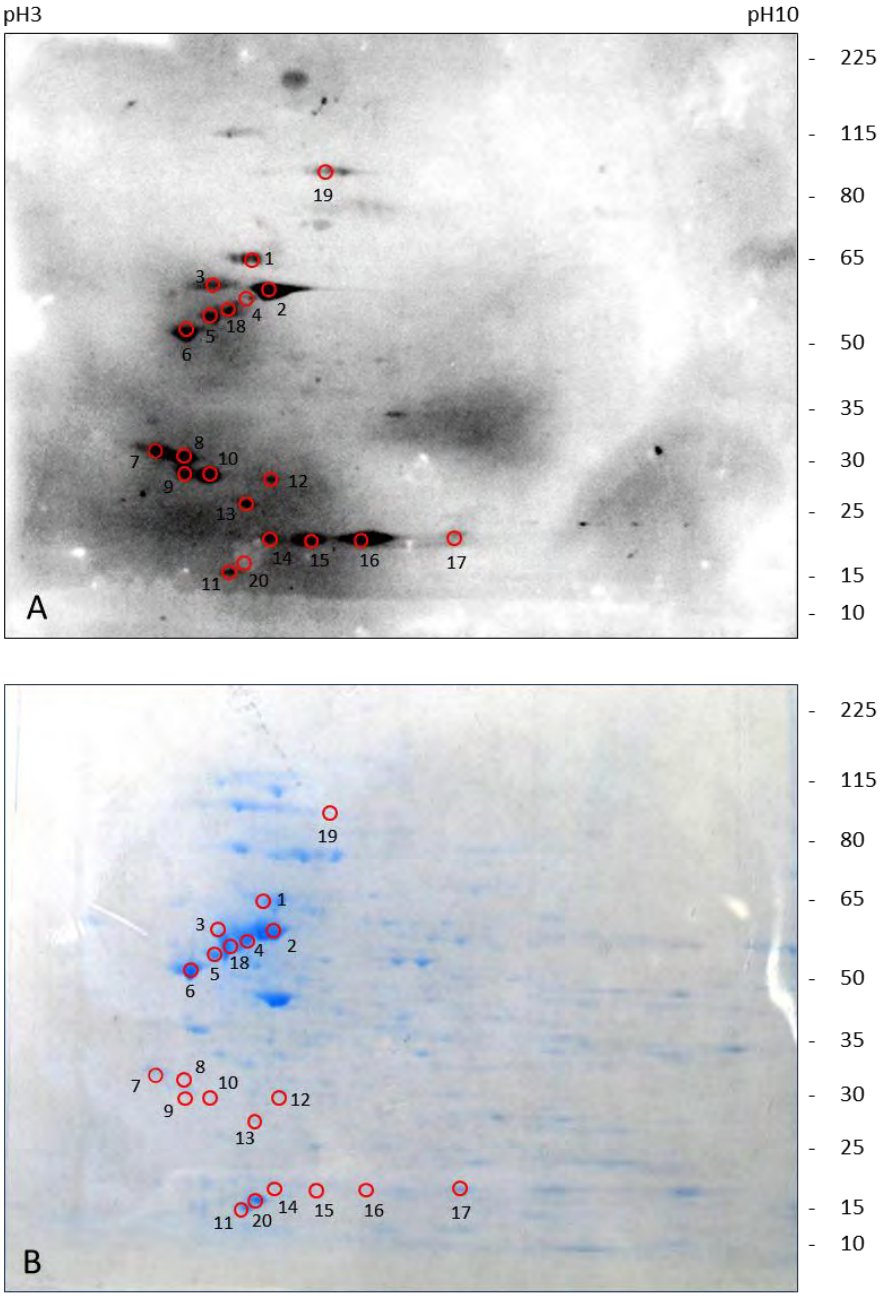

Figure 5.5 DFTD tumour-associated antigens selected for further identification by mass spectrometry. Detected protein spots in the 2D blot using sera from immunised devil 1 (**A**) were matched to spots in duplicate coomassie blue G-250 stained gel (**B**). Twenty matched spots were excised and identified using mass spectrometry methods. Their identities are listed in Table 5.1.

Table 5.1 DFTD tumour-associated antigens identified using sera from immunised devils.

Spot	Sequence Mas (kDa)		Peptides		Amino Acid Coverage	Protein name
	Predicted	Actual	Read	Unique		
9	28	28	16	6	33.3%	14-3-3 protein beta/alpha
9	28	28	4	3	9.3%	14-3-3 protein eta
9	28	28	39	7	32.0%	14-3-3 protein gamma
9	28	27	12	4	25.7%	14-3-3 protein theta
9, 10	28	27	76	9	47.3%	14-3-3 protein zeta/delta
1	62	61	42	12	22.16%	60 kDa heat shock protein, mitochondrial
19	85	87	10	4	6.3%	Aconitate hydratase, mitochondrial
10	28	24	11	2	13.6%	ADP-sugar pyrophosphatase
2, 4, 18	~55	55	213	28	52.0%	ATP synthase subunit beta, mitochondrial
1	62	60	2	2	12.96%	Beta-1-syntrophin
12	27	37	7	2	4.7%	Cathepsin B
11, 20	~15	15	167	11	48.2%	Cellular retinoic acid-binding protein
3	57	58	2	2	6.8%	Dachshund homolog 2
19	85	80	7	3	5.9%	DCC-interacting protein 13-alpha
19	85	82	19	4	7.3%	Gelsolin isoform 2
19	85	84	19	4	7.0%	Heat shock protein HSP 90-alpha
1, 2	~55	51	20	5	14.68%	Heterogeneous nuclear ribonucleoprotein K
14, 15, 16, 17	18	13	14	2	19.0%	Histone H2B type 1-F/J/L
18	54	53	18	9	16.9%	Perilipin-3
7	30	28	22	5	23.37%	Proliferating cell nuclear antigen
3	57	57	23	7	12.4%	Protein disulfide-isomerase
10	28	23	41	6	36.3%	Rho GDP-dissociation inhibitor 1
16	18	17	22	5	34.9%	Stathmin
17	18	15	15	2	8.4%	Superoxide dismutase [Cu-Zn]
7	30	28	15	8	27.02%	Tropomyosin alpha-4 chain-like isoform 4
2, 4, 18	~55	50	375	23	48.1%	Tubulin alpha-1B chain
4, 18	~55	49	424	28	49.1%	Tubulin beta chain-like isoform 1
18	54	49	31	4	12.1%	Tubulin beta-2A chain
4, 18	54	49	45	6	16.0%	Tubulin beta-2C chain
18	54	50	4	2	3.8%	Tubulin beta-3 chain
18	54	50	91	13	23.5%	Tubulin beta-5 chain
18	54	45	5	3	9.1%	Ubiquitin-like modifier-activating enzyme 5
1, 2, 3, 4, 5, 6, 18	~55	53	525	45	55.5%	Vimentin

Table 5.2 Summary of biological relevance of selected DFTD antigens identified by mass spectrometry analyses

Predicted protein
14-3-3 protein **Function:** **Contributes to cell cycle regulation, cell growth, differentiation, survival, apoptosis, migration and spreading.** **Associations with cancer:** **14-3-3 beta protein is involved in human gastric cancer progression and has potential as diagnostic and prognostic biomarker. 14-3-3zeta protein is currently undergoing extensive investigation as a novel therapeutic target. 14-3-3 theta protein has been identified as an antigen that induces a humoral response in lung cancer.** **References:** **(Freeman and Morrison, 2011, Fu *et al.*, 2000, Mhawech, 2005, Pereira-Faca *et al.*, 2007, Tseng *et al.*, 2011, Yang *et al.*, 2012, Zhao *et al.*, 2011).**
60 kDa heat shock protein (HSP60), mitochondrial **Function:** **Protein mostly localized in the mitochondrial matrix and outer mitochondrial membrane, constitutively expressed under normal condition, and induced by heat shock, mitochondrial damage, and mitochondrial DNA depletion.** **Associations with cancer:** **Overexpression reported in various cancers such as adrenal tumours and human breast, large bowel, bronchial, exocervical, ovarian and prostate cancers. It may have both pro and antiapoptotic roles in tumour cells. Serum antibodies against HSP60 are elevated in patients with osteosarcoma.** **References:** **(Cappello, 2003, Cappello *et al.*, 2003a, Cappello *et al.*, 2003b, Ciocca and Calderwood, 2005, Faried *et al.*, 2004, Hansen *et al.*, 2003, Lebret *et al.*, 2003, Pignatelli *et al.*, 2003, Trieb *et al.*, 2000, Wu *et al.*, 2009).**
Aconitate hydratase, mitochondrial **Function:** **Enzyme localised to the mitochondrion that catalyses the interconversion of citrate to isocitrate via cis-aconitate in the second step of the TCA cycle.** **Associations with cancer:** **Plays a key role in cellular bioenergy production and cell proliferation of human prostate carcinoma cells. Increase in the enzyme activity has been observed in malignant prostate cells.** **References:** **(Juang, 2004, Singh *et al.*, 2006).**
ATP synthase subunit beta, mitochondrial **Function:** **Enzyme that catalyses ATP synthesis. Localised outside of the cell membrane.** **Associations with cancer:** **Ectopic (outside the cell membrane) the enzyme has been proposed as a marker for targeted tumour therapy. The downregulation of the catalytic subunit of the enzyme is a hallmark of most human carcinomas.** **References:** **(Cuezva *et al.*, 2004, Lopez-Rios *et al.*, 2007, Ma *et al.*, 2010, Willers *et al.*, 2010).**

Predicted protein
Cathepsin B **Function:** **The protein is a lysosomal cysteine proteinase. It is also known as amyloid precursor protein secretase and is involved in the proteolytic processing of amyloid precursor protein (APP).** **Associations with cancer:** **The expression and subcellular localization of cathepsins change during cancer progression and cathepsins are involved in various aspects of tumourigenesis including metastasis and aggressive behaviour. Cathepsin B has been proposed as potential biomarker and therapeutic target in human cancers such as breast, human pancreatic ductal adenocarcinoma (PDA), cervical, colon, endometrial and pancreatic cancers.** **References:** **(Chan *et al.*, 2010, Devetzi *et al.*, 2009, Gopinathan *et al.*, 2012, Watson and Kreuzaler, 2009, Wu *et al.*, 2012)**
Cellular retinoic acid-binding protein 1 **Function:** **Specific binding protein for vitamin A and is thought to play an important role in retinoic acid-mediated differentiation and proliferation processes.** **Associations with cancer:** **Overexpressed in ovarian carcinoma tissues.** **References:** **(Hibbs *et al.*, 2004, Turhani *et al.*, 2006) http://www.ncbi.nlm.nih.gov/gene/1381**
DCC-interacting protein 13-alpha **Function:** **Protein involved in the regulation of cell proliferation, apoptosis, cell survival, endosomal trafficking, and chromatin remodelling.** **Associations with cancer:** **Protein associated with the molecular pathway that promotes ovarian cancer cell proliferation. Its function as pro-survival or pro-apoptotic factor in cancer cells is still controversial.** **References:** **(Deepa and Dong, 2009, Liu *et al.*, 2002, Tan *et al.*, 2010, Zhao *et al.*, 2010).**
Gelsolin isoform 2 **A ubiquitous actin-binding protein that plays a critical role in regulating ability of cells to migrate.** **Associations with cancer:** **The gelsolin gene is downregulated in multiple neoplasms and capable of suppressing tumourigenicity. Overexpression may be associated with progression of lung adenocarcinoma and is a potential target for therapy and as biomarker for lung adenocarcinoma. Gelsolin plays an important role in cellular proliferation and migration in cervical cancer and is also a promising marker for cervical cancer screening and prognosis** **References:** **(Gordon *et al.*, 2005, Li *et al.*, 2010, Liao *et al.*, 2011, Rodriguez-Pineiro *et al.*, 2010, Shao *et al.*, 2011).**

Predicted protein
Heat shock protein HSP 90-alpha **Function:** **HSP90 proteins are highly conserved molecular chaperones that have key roles in signal transduction, protein folding, protein degradation, and morphologic evolution.** **Associations with cancer:** **HSP90 interacts with a great number of molecules that are involved in the development and/or survival of cancer cells. HSP90 inhibition offers the potential of accomplishing the simultaneous disruption of multiple signalling events critical to tumour cell growth and survival. Phase II clinical trials are undergoing.** **References:** **(Cullinan and Whitesell, 2006, Jego *et al.*, 2010, Khalil *et al.*, 2011, Modi *et al.*, 2011, Mojtahedi *et al.*, 2011, Romanucci *et al.*, 2012, Sankhala *et al.*, 2011, Sidera and Patsavoudi, 2008, Whitesell and Lindquist, 2005).**
Heterogeneous nuclear ribonucleoprotein **Function:** **Transcription factor and role during cell cycle progression.** **Associations with cancer:** **This protein has been implicated in tumourigenesis. It was found overexpressed in melanoma and colorectal, oral, lung, nasopharyngeal, pancreatic, prostate and liver cancers.** **References:** **(Barboro *et al.*, 2009, Carpenter *et al.*, 2006, Du *et al.*, 2010, Jhaveri *et al.*, 2012, Michelotti *et al.*, 1996, Roychoudhury and Chaudhuri, 2007, Zhou *et al.*, 2010).**
Perilipin-3 **Function:** **Protein required for endosome-to-Golgi transport.** **Associations with cancer:** **Protein strongly expressed in invasive tumours, lymph node metastasis in cervical dysplasia and invasive carcinoma.** **References:** **(Muthusamy *et al.*, 2006, Szigeti *et al.*, 2009)**
Proliferating cell nuclear antigen **Function:** **Proliferating cell nuclear antigen (PCNA) is a cell cycle marker protein. It is well known as a DNA sliding clamp for DNA polymerase delta and as an essential component for eukaryotic chromosomal DNA replication and repair.** **Associations with cancer:** **PCNA is highly expressed in proliferating cells, including cancer and it is associated with enhanced malignancy. PCNA overexpression is correlated with cancer virulence. Its contribution to the cancerous state is mediated by its function in the processes that are necessary for tumour survival, such as DNA replication, repair of DNA damage, chromatin structure maintenance, chromosome segregation, and cell cycle progression** **References:** **(Chen *et al.*, 2011, Kannouche and Lehmann, 2004, Naryzhny and Lee, 2007, Naryzhny, 2008, Rosental *et al.*, 2011, Stenner *et al.*, 2012, Stoimenov and Helleday, 2009)**

Predicted protein
Protein disulfide-isomerase **<u>Function</u>:** **This enzyme is involved in hydroxylation of prolyl residues in preprocollagen. It also catalyses the formation, breakage and rearrangement of disulphide bonds.** **<u>Associations with cancer:</u>** **This protein is upregulated in breast cancer and implicated in melanoma progression.** **<u>References:</u>** **(Carta *et al.*, 2005, Hirata *et al.*, 2007, Zhang *et al.*, 2005).**
Rho GDP-dissociation inhibitor 1 **<u>Function</u>:** **Downregulator of Rho family GTPases. It prevents nucleotide exchange and membrane association.** **<u>Associations with cancer:</u>** **The expression of this protein is altered in a variety of cancers including oral squamous cell carcinoma and colorectal cancer. Overexpression of this protein promotes cell motility and lymph node metastasis. Higher frequency of autoantibodies against Rho-GDP proteins was found in nasopharyngeal and acute leukaemia patients.** **<u>References:</u>** **(Chiang *et al.*, 2011, Cui *et al.*, 2005, Dovas and Couchman, 2005, Harding and Theodorescu, 2010, Xiao *et al.*, 2007, Zhao *et al.*, 2010).**
Stathmin **<u>Function</u>:** **Stathmin is a member of a family of microtubule-destabilizing proteins that regulate the dynamics of microtubule polymerization and depolymerisation.** **<u>Associations with cancer:</u>** **Stathmin is overexpressed across a broad range of human malignancies including leukaemia, lymphoma, neuroblastoma, ovarian, prostatic, breast and lung cancers and mesothelioma. Stathmin is a potential target in cancer therapies that disrupt the mitotic apparatus.** **Stathmin is also upregulated in normally proliferating cell lines. In normal cells, stathmin is upregulated in neurons and anterior pituitary cells. In glial cells, stathmin is a constituent of the myelin sheath.** **<u>References:</u>** **(Belletti and Baldassarre, 2011, Cassimeris, 2002, Ghosh *et al.*, 2007, Gould *et al.*, 2008, Mistry *et al.*, 2005, Rana *et al.*, 2008, Rubin and Atweh, 2004, Zhang *et al.*, 2006)**
Tubulin **<u>Function</u>:** **Tubulin is an integral component of microtubules. It occurs mostly as soluble heterodimers consisting primarily of α- and β-tubulin isoforms or as assembled tubulin polymers that form microtubules.** **<u>Associations with cancer:</u>** **Autoantibodies against tubulin-alpha and tubulin-beta were detected in sera of renal and oral cell carcinoma and chronic myeloid leukaemia patients.** **<u>References:</u>** **(Kellner *et al.*, 2002, Luduena, 1998, Prasannan *et al.*, 2000, Shukla *et al.*, 2007, Zou *et al.*, 2005).**

Predicted protein
Vimentin **<u>Function</u>** **Vimentin is a major constituent of the intermediate filament family of proteins. It is ubiquitously expressed in normal mesenchymal cells; it helps maintaining cellular integrity and provides resistance against stress.** **<u>Associations with cancer:</u>** **Vimentin is overexpressed in various epithelial cancers, including prostate cancer, gastrointestinal tumours, tumours of the central nervous system, breast cancer, malignant melanoma, and lung cancer. Overexpression in cancer correlates well with accelerated tumour growth, invasion, and poor prognosis. Autoantibodies against vimentin were detected in sera from patients with pancreatic cancer. Anti-vimentin therapeutic approaches have also been proposed.** **<u>References:</u>** **(Hong *et al.*, 2006, Kokkinos *et al.*, 2007, Lahat *et al.*, 2010, Satelli and Li, 2011).**

5.4 Discussion

The identification of tumour-associated antigens (TAAs) eliciting humoral responses has been an important tool for understanding biological aspects of carcinogenesis and the interactions between cancer cells and the immune system (Ciocca and Calderwood, 2005). In humans, more than one hundred TAAs have been identified and several have been useful in diagnosis, prognosis and immunotherapy in various types of cancer (Buonaguro *et al.*, 2011). Based on the strategies applied in human models, the objective of this chapter was to use an immunoproteomic approach for the investigation of TAAs inducing a humoral response in Tasmanian devils immunised with non-viable DFTD cells.

The strategy implemented involved a combination of techniques that included two- dimensional electrophoresis coupled with western blot analysis, immunodetection and mass spectrometry (MudPIT). This proteomic approach is broadly known as SERPA (serological proteome analysis).

MudPIT is a method for rapid and large-scale proteome analysis by multidimensional liquid chromatography, tandem mass spectrometry (MS) and database searching. By exploiting a peptide's unique physical properties of charge and hydrophobicity, complex mixtures can be separated prior to sequencing by tandem MS. Tryptic digest is used for peptide fragmentation. This data is then used to identify the peptides are hence the proteins from which they are derived (Washburn *et al.*, 2001). In the MudPIT approach, the mass of a single peptide is measured to such a high degree of mass accuracy that the peptide is "unique" at that mass in the theoretical unique proteome of an organism (Cargile and Stephenson, 2004).

Non-unique tryptic peptides may affect the accuracy of accurate mass-based identification. This could include modified peptides, non-tryptic peptides (generated from cellular proteases), unpredicted/annotated peptides and other non-peptides sources (chemical noise). The specificity of the accurate mass approach is defined as the percentage of random noise that does not match unique tryptic peptides. Thus, specificity is equal to 100% minus the false positive rate. The false positive rate for protein (FPRprotein) identification is simply the combined probability that all the

peptides identifications for that protein are false for the number of peptides of a specific protein. The FPRprotein identification is a function of the false positive rate of peptide identifications (FPRpeptide) and the number of peptides used to identify a protein. It is considered that the FPRprotein identification should be approximately 1% or less. Thus, a peptide false positive rate of 10% translates into an identification specificity of 90% and 99% for one- and two-peptides hits respectively (Cargile and Stephenson, 2004). As protein identified by single peptides exhibit higher false positive rates (Elias *et al.*, 2005), confident protein identification is generally restricted to those identified by two or more peptides (Kocharunchitt *et al.*, 2012).

In addition to the number of peptides identified, the degree of coverage of the protein by peptides may also be used as a measure to assess the validity of the identification. This is provided in terms of the percentage of amino acids in the protein covered by identified peptides. Amino acid coverage is particularly useful to increase the credibility of proteins identified by single peptides. However, it is important to note that, for a particular protein, only a portion of the peptides are actually observed experimentally by MS (Sanders *et al.*, 2007).

The molecular weight predicted for each protein was estimated form the SDS gels by comparison to pre-stained molecular weight markers. These markers runs differently than unstained proteins. Furthermore, factors such as protein structure, posttranslational modifications and amino acid composition are variables that can affect the electrophoretic migration of proteins. Therefore, the predicted molecular weight is better referred to as the apparent molecular weight. In the current research, proteins with an actual molecular weight of ± 10kDa the predicted molecular weight were included in the list of proteins identified.

Using the SERPA approach and the criteria of two or more unique peptides identified, 24 DFTD TAAs were preliminarily identified by this current study. Amino acid coverage ranged from 4.7% to 55.55% for the protein vimentin, which also had the highest number of unique peptides identified (55 peptides, see Table 5.1).

In humans, the presence of antibodies against tumour antigens in the serum of cancer patients is well documented (Reuschenbach *et al.*, 2009). Although some of the immune

responses in cancer patients recognize antigens that are found only in tumours, most antibodies are directed against self-antigens (Tan *et al.*, 2009). This is because most cancer antigens are derived from mutated or modified self-proteins. These proteins are the product of the accumulation of several genetic modifications in somatic cells, which in turn provide cancer cells with the advantage of growth and clonal expansion during the tumourigenesis process (Pardoll, 2003, Vogelstein and Kinzler, 2004).

How the TAAs elicit an immune response is not completely clear, especially as many described TAAs are intracellular proteins (Reuschenbach *et al.*, 2009, Zinkernagel, 2000). As the immunised devils received total cell extract, intracellular antigens would be released and the more immunogenic would induce a response. Antibodies to these intracellular antigens have been detected in the cancer process, thus they are likely to be true TAAs. It is believed that antibodies against these antigens develop as part of the natural immune response to the changes in protein structure and expression (Tabernero *et al.*, 2010). These changes include protein modifications (point mutations or post-translational modifications) or altered tissue expression (Fonseca and Dranoff, 2008, Houghton, 1994, Vesely *et al.*, 2011). The overexpression of these tumour-associated antigens may result in a level of expression above the threshold for T cell recognition, breaking the immunological tolerance to self-antigens and triggering an anti-cancer response (Buonaguro *et al.*, 2011). Another hypothesis suggests that aberrant tumour cell death such as necrosis can expose modified intracellular proteins, which are presented to the immune system in an inflammatory environment (Fernandez Madrid, 2005, Tan *et al.*, 2009).

The DFTD antigens identified in this study are proteins with a wide variety of functions that may provide clues about the tumourigenesis processes occurring in devil facial tumour cells. They act in pathways that have been identified as commonly mutated in cancer (or "hallmarks of cancer cells" as described by Hanahan and Weinberg (2011)). These pathways include cell cycle progression (14-3-3 proteins), signal transduction (HSP90 and HSP60), cellular bioenergy production (aconitate hydratase and ATP synthase), proliferation (14-3-3 proteins, cathepsin B, cellular retinoic acid, gelsolin and proliferating cell nuclear antigen), migration (gelsolin and cathepsin B) and apoptosis (14-3-3 protein, HSP60 and DCC-interaction protein 13-alpha). Future studies are

required to confirm differential or aberrant expression of these proteins in devil facial tumour cells compared to normal tissues. Strategies to undertake this protein expression studies (e.g. immunohistochemistry or flow cytometry) or gene expression studies (e.g. quantitative PCR).

A single protein, identified as stathmin induced an antibody response in both immunised devils. This is of particular importance because it is likely that different animals may recognise this DFTD antigen, which is crucial for the development of a vaccine. Although these are preliminary studies using sera from just two animals, it is a promising finding for future research.

Stathmin may have a role in the development of DFTD cancer cells. The protein is a member of a family of microtubule-destabilizing proteins that regulate the dynamics of microtubule polymerization and depolymerisation. It was initially classified as an "oncogene" because mutations of the gene can cause uncontrolled cell proliferation as seen in cancer cells (Cassimeris, 2002). Whether mutations or structural alterations of the gene occur in DFTD it would be worthy of investigation.

Antibodies against proteins such as vimentin, 14-3-3 proteins, and heat shock proteins have been found in a variety of human cancers (Table 5.2). These antigens have been the subject of extensive investigations as therapeutic targets for cancer and some clinical trials are underway because they participate in several pathways required for cancer cell survival, proliferation, and migration (Lahat *et al.*, 2010, Sankhala *et al.*, 2011, Yang *et al.*, 2012). Their relevance in immunotherapy relates to the possibility of targeting multiple pathways simultaneously. These proteins could be also useful as anti-cancer therapeutic targets in DFTD.

Antibody reactivity against few DFTD proteins was also detected in the pre-immune sera and sera from naïve devils. Similar findings have been reported in humans. Studies have showed that a considerable percentage of the sera of healthy subjects contain autoantibodies against several self-antigens. The studies proposed that these antibodies are produced in responses to non-malignant disorders (Li *et al.*, 2006, Nolen *et al.*, 2009). This notion highlights the importance of including as many control samples as

possible (serum from naïve animals) in order to discriminate cancer-specific antigens from those not associated with tumour development.

The opportunity to specifically identify and differentiate the antibody reactivity in sera from immunised devils from that of naïve individuals (i.e. by identifying cognate antigens) is also important because this is not currently possible using techniques such as flow cytometry and ELISA. Therefore, it is possible that spontaneous natural responses to DFTD (if they occur) could be better characterised using this approach.

Initial flow cytometry studies from our group using intact DFTD cells and the sera from immunised devils allowed the detection of antibodies against cell surface antigens (data not shown). However, only intracellular proteins were identified in this study. This could be related to the use of 2DE for the separation of DFTD proteins. This technique is still considered the best method for the high-resolution separation of a complex mixture of proteins and the identification of protein isoforms (Tan *et al.*, 2009). These advantages were observed in this study (e.g. five isoforms for 14-3-3 proteins were identified and five isoforms for tubulin beta). However, one of the main drawbacks of 2DE is the limited separation of hydrophobic proteins and low abundant proteins (Zou *et al.*, 2005). It is likely that DFTD membrane proteins were poorly represented in the samples. Specialised kits are available to increase the yield of non-soluble proteins during sample preparation. Incorporation of these techniques to this approach might allow to identification of cell surface TAAs.

Although only preliminary, the results from this chapter highlight the feasibility of applying an immunoproteomic approach for the detection of DFTD TAAs using the sera from immunised devils. The characterization of these antigens has the potential to open new areas of research to increase our knowledge about the biology of the DFTD cancer cells. It may also provide opportunities for the development of a vaccination approach to protect devils against this cancer where alternative approaches are desperately required.

6 General discussion

General Discussion

DFTD is a contagious cancer that is transmitted as an allograft between related and unrelated individuals. Recent cytogenetic and molecular analyses have confirmed that the infectious agent is the cancer cell itself (Miller *et al.*, 2011, Pearse and Swift, 2006, Siddle *et al.*, 2007b). There is only one other known case of a naturally occurring transmissible cancer, canine transmissible venereal tumour (CTVT) of domestic dogs. CTVT tumours are also transmitted as an allograft and are maintained in the dog population by coitus and other social behaviours (Karlson and Mann, 1952). CTVT is a benign cancer where the tumours generally regress within six months (Das and Das, 2000). Therefore, CTVT evolved to be a successful parasite in dogs even though they are capable of mounting an effective immune response against the tumours. In contrast, DFTD is an aggressive and invariably fatal cancer. Affected animals die within months of tumour appearance, generally from starvation due to the size and facial location of the tumours or by organ failure due to metastases (Pyecroft *et al.*, 2007).

There is no evidence for an effective natural immune response in the Tasmanian devils to the transfer, establishment and spread of DFTD tumour cells. This total lack of allograft response is, therefore one of the most intriguing aspects of this cancer. The immune system plays an important role in the defence against allografts and cancer cells (Vesely *et al.*, 2011). Downregulation of MHC-I in cancer cells is a common mechanism of immune evasion and explains the transplantation of allogeneic cells in CTVT (Karlson and Mann, 1952). In contrast, our knowledge of DFTD cells is limited and the mechanisms of immune evasion are not yet understood. In regards to MHC-I expression in Tasmanian devils and DFTD cells, this has only been investigated at the genetic level. Therefore, this thesis focused on studying various aspects (including MHC-I) of devil facial tumour cell in order to elucidate the origin of the cells and the mechanisms involved in the immune evasion by this transmissible cancer.

Schwann cell origin of devil facial tumour disease

Results of this thesis provide significant evidence supporting a peripheral nerve origin of devil facial tumour cells. Previous genetic studies of the tumour transcriptome indicated that the cancer cells express several genes related to the myelination pathway

in the peripheral nervous system (Murchison *et al.*, 2010). Results from Chapter 2 demonstrated that the tumour cells express a range of myelin and other peripheral nerve proteins. Thus, the combined genetic and protein expression support the hypothesis that devil facial tumour cells are of Schwann cell origin. This is consistent with the proposed neuroectodermal nature of the tumour (Loh *et al.*, 2006b). Furthermore, the expression of markers of highly differentiated Schwann cells provided additional evidence that the cancer cells are more likely to be of the peripheral nerve lineage rather than neuroendocrine as originally proposed (Loh *et al.*, 2006b).

There is known association between cancer and chronic injury (Coussens and Werb, 2002). The features of Schwann cells may provide insights into their transformation to cancer cells during chronic injury in Tasmanian devils. Mature Schwann cells are extremely plastic and play an active role during nerve repair. After nerve injury, myelin proteins are downregulated and the cells regain the potential to proliferate. These cells then re-differentiate and re-myelinate regenerated axons as part of the repair process (Mirsky *et al.*, 2008, Parkinson *et al.*, 2008). Devil bites produce frequent injury and infection in areas highly innervated such as the devils' vibrissae. Cells that participate in tissue regeneration, including Schwann cells, have an innate capacity of self-renewal, unlimited replication and the cells are relatively long-lived in comparison to other cells within the tissues (Beachy *et al.*, 2004). This makes these cells particularly susceptible to the carcinogenic effects inflammatory mediators, such as free radicals, prostaglandins, and cytokines. Prolonged exposure to these factors promotes cell proliferation, mutagenesis, oncogene activation and angiogenesis (Shacter and Weitzman, 2002). Moreover, migration through tissues, which is a normal feature of neural crest cells and has been observed during repair in adult tissues, is a characteristic of cancer invasion and metastases (Beachy *et al.*, 2004, Wilson and Gibson, 1997). Thus, the remarkable ability of Schwann cells for repair may actually predispose them to tumourigenesis resulting in the formation of aggressive tumours. Therefore, repeated tissue injury caused by aggressive, but natural devil interactions, may have contributed to the development of a facial malignant Schwann cell neoplasm in Tasmanian devils.

The demonstration that DFTD cells consistently express proteins of highly specialised Schwann cells led to the significant discovery that periaxin, a myelin protein of the

peripheral nervous system, is a sensitive and reliable marker for the diagnosis of DFTD tumours in histology samples (chapter 3). There is a clear advantage of using this method for diagnosis compared to cytogenetics, as cytogenetic diagnosis requires collection of fresh samples and the establishment of cell cultures. Furthermore, periaxin is particularly useful for distinguishing DFTD from other common devil neoplasms or biting wounds, and its use will facilitate monitoring of the disease in the field.

The identification of periaxin as a marker of DFTD during this study may have broader implications in cancer diagnosis. Peripheral nerve sheath tumours have also been described in humans and other mammals and studies suggest that most of these neoplasms, both benign and malignant, are derived from Schwann cells (Carroll and Ratner, 2008, Zhu *et al.*, 2002). Currently, identification of these tumours lacks precision, as there is no known definitive marker. Therefore, periaxin represents an excellent candidate marker for diagnosis of peripheral nerve sheath tumours in other species.

Identification of the Schwann cell origin of devil facial tumour cells gives insights into possible mechanisms of immune evasion by this transmissible cancer

The constitutive levels of MHC-I protein expression across normal devil tissues were found to be similar to reported levels in matched tissues obtained from humans and other species. Devil spleen cells, lymph nodes and cells within the skin expressed high levels of MHC-I protein, whereas peripheral nerve tissue cells expressed low levels of MHC-I. Upon closer analysis of MHC-I expression in devil tissue, results of this thesis showed that in normal cells MHC-I expression was clearly associated with the cell membrane. This is of much significance as it demonstrates that the complex machinery required for MHC-I translation, assembly and trafficking is functional in the Tasmanian devil. This provides further evidence that Tasmanian devils have the necessary components of a competent immune system.

In humans and other species, low levels of MHC-I expression in Schwann cells have been associated with the notion of immune privilege and protection given by the blood-nerve interface in the peripheral nervous system (Armati *et al.*, 1990, Forrester *et al.*, 2008, Tsuyuki *et al.*, 1998, Weerasuriya and Mizisin, 2011). Since the cancer cells in

DFTD are most likely derived from a Schwann cell, it was not surprising to find evidence that they also have low MHC-I expression. However, even a low level of expression in the DFTD cancer cells, which are exposed to the immune system, should provide a sufficient signal for immune recognition.

A further significant observation during this study was that MHC-I was detected in the perinuclear region of devil facial tumour cells, with no evidence of MHC-I expression at the cell surface. The lack of antibodies specific to an external epitope of devil MHC-I meant that several methods were employed to verify the absence of MHC-I membrane expression. The most convincing evidence was confocal microscopy. The results from this work found no evidence for MHC-I expression at the cell surface. This was despite the presence of intracellular MHC-I.

Recent preliminary experimental data from our work with collaborators at the University of Cambridge provided (during the writing of this thesis) extra evidence supporting the reduced expression of MHC-I at the cell surface of DFTD cells. Flow cytometry studies using a new polyclonal anti-devil beta-2 microglobulin (B2M) antibody showed that B2M was not detected at the surface of DFTD cells (A. Kreiss and H. Siddle, personal communication, December 2011). Furthermore, incubation of DFTD cells with a supernatant (containing cytokines) produced using mitogen stimulated devil leukocytes, induced B2M surface expression. Functional and stable MHC-I molecules are heterotrimers consisting of the MHC-I heavy chain, B2M and a peptide ligand, which are assembled within the endoplasmic reticulum (ER). Only stable MHC-I molecules are exported from the ER to the outer membrane (Flajnik and Kasahara, 2001). Thus, a reduced expression of B2M in devil facial tumour cells may contribute to a reduced trafficking of MHC-I to the cell surface.

An absence of MHC-I antigens presented at the cell surface of devil facial tumour cells would impede recognition of tumour cells and therefore inhibit T cell-mediated destruction. However, cells with a deficiency in expression of MHC-I should be selectively rejected by NK cells according to the classical theory of the "missing self" (Ljunggren and Karre, 1990). Recent models that complement this hypothesis show that NK cell triggering also requires the expression of inducible ligands of activating NK receptors (Diefenbach and Raulet, 2001). Therefore, the activation of NK cells depends

on a complex balance between activating and inhibitory signals (Lanier, 2005). T cell functionality in devils has been demonstrated by skin grafts and strong mixed lymphocyte reactions (Kreiss *et al.*, 2011a). On the contrary, detailed knowledge of the existence of a functional NK population is limited. Preliminary studies in devils immunised with devil facial tumour cells or the leukaemia human cell line K562 suggest that devils have NK cells capable of producing functional cytotoxic responses against xenogeneic cells. However, in this study, NK cells were not able to recognise DFTD cells (Brown *et al.*, 2011). Further investigation is required to study specific NK cell subpopulations and the expression of NK receptors that may mediate immune selection of cells deficient in MHC molecules.

Because Schwann cells constitutively express low levels of MHC-I it is likely that the limited surface expression of MHC-I molecules in DFTD is a trait associated with the cell of origin rather than a selective response of the tumour cells (i.e. post carcinogenic transformation) to avoid immune destruction. Devil facial tumour cells may also inherited the immune privilege that characterises Schwann cells. Thus, features characteristic to Schwann cells may either directly or indirectly play a role in the process of evolution of a somatic cell into not only a malignant tumour but also into a transmissible cancer. This notion has important implications because it provides new lines of research to understand the underlying mechanisms that regulate MHC-I expression in the DFTD cancer cells.

Studies in human cancer have shown that structural alterations in MHC-I genes (i.e. mutations or deletions) as a mechanism of immune evasion are rare (reviewed in Seliger, 2012). The more likely mechanism is de-regulated expression of MHC-I molecules and components of the antigen processing machinery (APM). This deregulation has been observed in several human cancers including peripheral nerve tumours (Ferris *et al.*, 2006, Kamphausen *et al.*, 2010, Lee *et al.*, 2004, Lee *et al.*, 2006, Lopez-Albaitero *et al.*, 2006). Deregulation may result from a variety of mechanisms, transcriptional, posttranscriptional or epigenetic. Although epigenetic changes such as methylation and histone modifications are common in cancer (Esteller, 2008), epigenetic modifications of APM components are much less common and the frequency of them occurring compares to that of mutation rates. More frequently, transcriptional

and/or posttranscriptional modifications occur (Seliger, 2012). Moreover, up-regulation of MHC-I and APM components can be mediated by cytokines, which restores their expression (Respa *et al.*, 2011, Rodriguez *et al.*, 2007). Studies in CTVT provide a model of the regulatory process controlling MHC-I expression. During the progression phase CTVT tumour cells produce high concentrations of TGF-β which decreases B2M and MHC-I expression (Cohen *et al.*, 1984). During the regression phase tumour-infiltrating lymphocytes secret IFN-γ and interleukin-6 (IL-6) which block the inhibitory effects of TGF-β and induce the expression of MHC-I in the tumour cells (Hsiao *et al.*, 2008).

Although knowledge of the mechanisms that regulate expression of MHC-I in Schwann cells is limited (Meyer zu Horste *et al.*, 2010), experimental studies suggest that modulatory pathways have a significant role in control. Normal Schwann cells constitutively express low levels of MHC-I molecules but this expression can be increased by pro-inflammatory cytokines such as IFN-γ (Armati *et al.*, 1990, Lilje and Armati, 1997). The finding that DFTD tumour cells express different levels of MHC-I proteins under different conditions suggests that modulatory pathways also function in these cells. These results together indicate that alteration of MHC-I expression in DFTD cells is unlikely to occur due to irreversible structural genetic changes and it is more plausible that other cellular regulatory mechanisms control MHC-I expression in these cancer cells.

MHC-I expression was shown to be restricted to the perinuclear region of the tumour cells suggesting that MHC-I molecules are retained at the ER compartment. This could be due to de-regulated expression of other components of the antigen processing pathway. Mechanisms of immune evasion by deregulation of APM components have been described in human cancers. For instance, a coordinated alteration in the expression of MHC-I,B2M, the proteasome subunit beta (LMP2), and the transporter associated with antigen processing (TAP1) was found in bladder cancer (Romero *et al.*, 2005). Our research group is currently in the process of studying the antigen presenting pathway and its role in DFTD by measuring gene expression of genes encoding B2M and the proteasome subunit, beta type, 8 (PSMB8, also known as LMP7). This proteasome is essential for the processing of MHC-I peptides (Fehling *et al.*, 1994).

Preliminary data shows reduced expression of both B2M and PSMB8 in primary devil facial tumour cells compared to cells of normal spleen tissue (Howson, 2011). Thus, both reduced MHC-I expression and reduced expression of components of the antigen-processing pathway may contribute to a significant lack of trafficking of MHC-I to the DFTD cell surface, ultimately resulting in a lack of immune recognition by $CD8^+$ T cells.

Preliminary data also shows that cell surface B2M expression in devil facial tumour cells could be recovered after cytokine treatment and this ability to re-establish surface B2M may be useful for the development of a DFTD vaccine. Studies in humans with normal and cancer cells have shown that cytokines such as IFN-β, IFN-γ, and TNF-α increase the coordinated expression of MHC-I and APM components (Epperson *et al.*, 1992, Liu *et al.*, 2011, Respa *et al.*, 2011). Therefore, it is feasible that using a similar approach, optimal immune responses against DFTD could be activated following induced upregulation of both MHC-I and associated APM components. This is the focus of current DFTD research.

Alteration of MHC-I and APM components are only one aspect of the different strategies that tumours can use to escape immune cell surveillance (Stewart and Abrams, 2008). Tumours can also secrete immune suppressive factors, activate negative regulatory pathways and alter the cellular mechanisms of signal transduction. Any single or combined strategy would suppress either directly or indirectly, innate or adaptive immune responses, and therefore impair an effective anti-tumour immune response (Seliger, 2012 , Stewart and Abrams, 2008). Therefore it follows that study of the occurrence of additional mechanism of immune evasion by DFTD are required, as well as investigation of the functionally of other immune cells (such as NK cells and dendritic cells) that participate in the immune surveillance against tumours.

Additionally, interleukins and other factors secreted by tumour cells can induce immune suppression and favour tumour progression. This has been observed during the establishment of CTVT tumours. The cancer cells produce high concentrations of TGF-β, which decrease MHC-I expression, suppress NK cell activity, and prevent dendritic cell maturation and activation (Hsiao *et al.*, 2004, Liu *et al.*, 2008). Preliminary studies in our laboratory detected both transforming growth factor beta

(TGF-β) and interleukin-10 (IL-10) in the DFTD tumour microenvironment. These studies also showed that although mature dendritic cells were identified in lymph nodes, only immature dendritic cells were observed surrounding DFTD tumours (Howson, 2011). The combined actions of low MHC-I expression and secretion of immune suppression factors may together be responsible for the lack of effective immune responses to DFTD and this area requires further investigation.

Devil facial tumour-associated antigens may provide a tool for the development of a vaccine

The emergence of DFTD caused great concern about the possibility of losing the largest marsupial carnivore and Tasmanian icon. Conservation management strategies were promptly established. They included the removal of DFTD-affected devils from relatively isolated local populations in Tasmania and the establishment of insurance populations of healthy devils in mainland Australia and in Tasmanian free-range enclosures (http://www.tassiedevil.com.au/tasdevil.nsf).

Evaluation of control measures such as selective culling of infected individuals has been ineffective (Lachish *et al.*, 2010) and results from this thesis indicate that DFTD cells have mechanisms that allow immune escape. Thus, the original hope of finding resistant animals in the wild is rapidly becoming implausible. Vaccination is therefore an important and feasible strategy for the management and conservation of the species (Jones *et al.*, 2007, Woods *et al.*, 2007).

While routine vaccination aims to induce a lasting immune response that prevents infectious diseases (Ada, 2005, Lombard *et al.*, 2007, W.H.O., 2008), vaccination applied to cancer focuses on methods that enhance the avidity of MHC-I restricted T cells in order to overcome the relatively poor antigen presentation in tumours (Rosenberg, 2001).

Chapter 5 of this study presented the development of an immunoproteomic approach for the identification of tumour-associated antigens (TAAs) in devil facial tumour cells. Using this approach, more than 20 preliminary TAAs were identified. An advantage of

characterizing TAAs in DFTD compared to similar strategies in humans (where the heterogeneity of tumour cells is high, even among patients affected by the same type of cancer) is the clonal nature of the tumours and the stability of the DFTD genome (Murchison *et al.*, in press). This means that tumour antigens are more likely to be conserved and that the immune response will have a constant antigenic target, aspects that are essential for the development of a vaccination approach (Woods *et al.*, 2007).

In human cancer, humoral responses develop as part of the immune response to changes in protein structure and/or expression (Tabernero *et al.*, 2010). These proteins are likely to have a role in tumour growth by regulating processes such as cell proliferation, angiogenesis and tumour invasion. Therefore, the identification of these antigens in devil facial tumour cells is also relevant for the understanding of underlying carcinogenesis processes. Examples of the DFTD TAAs identified include the 14-3-3 protein and HSP-90, which contribute to regulating the cell cycle, survival and migration; aconitate hydratase protein which plays an important role in cellular bioenergy production; and cathepsin B that has been involved in cancer progression and metastases. The fact that these processes have been considered hallmarks of cancer (Hanahan and Weinberg, 2011), indicates that these mechanisms may play an important role in the development of devil facial tumours.

In humans, the identification of antigens has also been useful for the development of immunotherapeutic approaches. Active vaccination attempts to elicit a specific host immune response against selected tumour antigens. It focusses on strategies that activate $CD8^+$ T cells because they can recognise peptides derived from endogenously expressed proteins (Hirohashi *et al.*, 2009). Cytotoxic T lymphocytes can recognise all tumour-associated antigens and therefore have the potential to directly kill their target cell (Poschke *et al.*, 2011). These new strategies use a combination of approaches that trigger the innate immune system and adaptive humoral and cellular immunity. Approaches already adopted include whole tumour proteins containing multiple and relevant antigenic epitopes (Jager *et al.*, 2000); vaccines using cytokines as adjuvants (Hara *et al.*, 2000, Litzinger *et al.*, 2007); and dendritic cells loaded with peptides or proteins *in vitro* (Osada et al., 2006).

A significant and promising result of this preliminary research of DFTD TAAs is that one antigen was recognised by immune sera from two different devils. The presence of common antigens is crucial for the development of a vaccination strategy because it is likely that they will evoke an immune response in all affected devils. The antigen identified was stathmin and this protein is relevant for targeted immunotherapy because of its role in the regulation of the dynamics of microtubule polymerization and depolymerisation (Cassimeris, 2002, Rubin and Atweh, 2004). Stathmin is overexpressed across a broad range of human malignancies including leukaemia, lymphoma, neuroblastoma, ovarian, prostatic, breast and lung cancers and mesothelioma (Ghosh *et al.*, 2007, Mistry *et al.*, 2005, Rana *et al.*, 2008) and it is considered a potential target in cancer therapies that disrupt the mitotic apparatus (Belletti and Baldassarre, 2011, Zhang *et al.*, 2006). It is now also a possible target for DFTD chemotherapy.

The results of this thesis have made a significant contribution not only to the understanding of DFTD and to the mechanisms of tumourigenesis and immune invasion but have also identified proteins with potential for use in vaccine development and targets for chemotherapy.

Summary

The research presented in this thesis has provided significant advances in our knowledge of the biology and pathology of devil facial tumour disease. It confirmed the neuroectodermal nature of the cancer and provided strong evidence indicating that the disease is a malignant peripheral nerve sheath tumour of Schwann cell origin.

The identification of the Schwann cell as the cell of origin contributed to the discovery of periaxin as a sensitive and specific marker for the cancer that has improved diagnosis in histology samples. From these results, periaxin could provide a promising diagnostic marker for peripheral nerve tumours in other species, especially humans.

A Schwann cell origin of DFTD is relevant for the understanding of the emergence and spread of devil facial tumour cells. The plasticity of Schwann cells and their role in nerve tissue repair may have contributed to the development of facial tumours. This relates to the characteristic biting behaviour of the Tasmanian devils and the concept that chronic inflammation promotes carcinogenesis in plastic cells.

This research also contributed to the notion that Tasmanian devils are immunocompetent. It was found that cells in normal devil tissues synthesise MHC-I proteins which are expressed at the cell surface. This suggests that normal cells have the functional machinery to transport cellular antigens to surface and present them to the immune system.

More importantly, this thesis provided evidence that downregulation of surface MHC-I expression is a possible mechanism used by devil facial tumour cells to avoid immune detection. Tumour cells expressed low levels of MHC-I proteins, which were not associated with the cell membrane. This provides devil facial tumour cells with a survival advantage, as they would be able to grow undetected by the immune system. Normal Schwann cells in peripheral nerve bundles also showed very low levels of MHC-I protein expression. Thus, the poor immunogenicity observed in devil facial tumour cells is likely to be a trait inherited from the cell of origin.

This thesis also showed that devil facial tumour associated antigens (TAAs) eliciting humoral immune responses in immunised devils can be detected using an immunoproteomic approach. These antigens are important for understanding the tumourigenesis process as many of the antigens detected have been characterised in a range of human cancers. These antigens offer potential immunological targets for a prospective vaccine strategy.

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8 Appendixes

Appendix 1

Publications

Appendix 1 has been removed for Copyright or Proprietary reason

Tovar C, Obendorf D, Murchison EP, Papenfuss AT, Kreiss A, Woods GM (2011). Tumor-specific diagnostic marker for transmissible facial tumors of Tasmanian devils: immunohistochemistry studies. *Veterinary Pathology* 48: 1195-1203

http://dx.doi.org/10.1177/0300985811400447

Kreiss A, **Tovar C**, Obendorf DL, Dun K, Woods GM (2011). A murine xenograft model for a transmissible cancer in Tasmanian devils. *Veterinary Pathology* 48: 475-481.

http://dx.doi.org/10.1177/0300985810380398

Murchison EP, **Tovar C**, Hsu A, Bender HS, Kheradpour P, Rebbeck CA *et al* (2010). The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally transmissible cancer. *Science* 327: 84-87.

http://dx.doi.org/10.1126/science.1180616

Appendix 2

List of Reagents

1. Reagents

Reagent	Supplier	Catalogue Number
2-Mercaptoethanol	Sigma-Aldrich	M6250
3-Aminopropyltriethoxysilane	Sigma-Aldrich	A3648
Acrylamide (30%)	Bio-Rad	161-0156
Antibody Diluent	Dako	S0809
APS	Bio-Rad	161-0700
Bromophenol Blue	Serva	15375
Coomassie Brilliant Blue G-250 Dye	Thermo Scientific	20279
DC™ Protein Assay	Bio-Rad	500-0111
Dispase	Invitrogen	17105-041
Di-sodium hydrogen phosphate	Merck	1.06586.0500
DTT	Invitrogen	15508-013
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	11965
EnVision+ System-HRP Labelled Polymer Anti-Mouse	Dako	K4005
EnVision+ System-HRP Labelled Polymer Anti-Rabbit	Dako	K4003
Ethanol	Merck	4102309020
Foetal Calf Serum	Gibco	1099-141
Fibroblast Growth Factor	Promega	G5071
FIX & PERM® reagents	Invitrogen	GAS003
Formalin, 10% Neutral Buffered	Sigma-Aldrich	F5554
Forskolin	Sigma-Aldrich	F6886
GelCodeTM Blue Safe Protein Stain	Thermo Scientific	24594
Gentamicin Sulphate	Pfizer	61022027
Glycerol	Sigma-Aldrich	G6279
Glycine	Sigma-Aldrich	G8898
Halt™ Protease Inhibitor Cocktail	Thermo Scientific	87786
Harris Haematoxylin	CliniPure	CP-L-HAEMHARR-06
Histopaque®-1077	Sigma-Aldrich	10771

Reagent	Supplier	Catalogue Number
Hydrogen Peroxide Solution	Sigma-Aldrich	216763
Immobilon Western Chemiluminescent HRP Substrate	Millipore	WBKLS0500
In-Gel Tryptic Digestion Kit	Thermo Scientific	89871
Iodoacetamide	Bio-Rad	163-2109
L-glutamine	Sigma-Aldrich	G7513
Liquid DAB+ Substrate Chromogen System	Dako	K3468
Methanol	Merck	901459020
Montanide Adjuvant Essai 70M10645101	Seppic	L 13206
N,N-dimethylacrylamide	Sigma-Aldrich	274135
NuPAGE® Antioxidant	Invitrogen	NP0005
NuPAGE® LDS Sample Buffer (4X)	Invitrogen	NP0007
NuPAGE® MOPS SDS Running Buffer (20X)	Invitrogen	NP0001
NuPAGE® Novex® 4-12% Bis-Tris Gels	Invitrogen	NP0322BOX
NuPAGE® Sample Reducing Agent (10X)	Invitrogen	NP0004
NuPAGE® Transfer Buffer (20X)	Invitrogen	NP0006-1
Paraformaldehyde	Sigma-Aldrich	P6148
Penicillin-Streptomycin	Sigma-Aldrich	P4333
PERTEX Mounting Medium	HD Scientific	CP MD 41-4012-00
Pituitary Extract bovine	Sigma-Aldrich	P1476
Poly-L-Lysine	Sigma-Aldrich	P-4832
Potassium chloride	Calbiochem	529552
Potassium phosphate monobasic	Sigma-Aldrich	P5655
Protease Inhibitor Cocktail	Thermo Scientific	87786
Protein Block Serum-Free	Dako	X0909
RIPA Buffer	Thermo Scientific	89901
RPMI 1640 Medium	Gibco	22400-089
Skim Milk Powder	Diploma	-
Sodium Chloride	Sigma-Aldrich	631014
SDS	Bio-Rad	161-0302
Spectra™ Multicolor Broad Range Protein Ladder	Fermentas	SM1841

Reagent	Supplier	Catalogue Number
Target Retrieval Solution	Dako	S1700
TEMED	Bio-Rad	161-0801
Tris-HCl	Merck	1.08219.1000
Trizma Base	Sigma-Aldrich	T1503
Triton™ X-100	Sigma-Aldrich	X100
Trypan Blue	Sigma-Aldrich	T6146
Trypsin Solution 10X	Sigma-Aldrich	59427C
Tween 20®	Sigma-Aldrich	P1379
Xylene	M & B Pronalys	L864
ZOOM® 2D Protein Solubilizer 1	Invitrogen	ZS10001
ZOOM® Carrier Ampholytes pH 3-10	Invitrogen	ZM0021

2. Disposables

Product	Supplier	Catalogue Number
0.1-20 µl Eptips	Eppendorf	022492012
0.45 µm Nitrocellulose Membrane	Bio-Rad	162-0094
1.0 mm zirconia/silica beads	Biospec Products	11079101z
15 ml Centrifuge Tube	IWAKI	2325-015
2-200 µl Eptips	Eppendorf	022492039
50-1000 µl Eptips	Eppendorf	022492055
70 µm Cell Strainer	BD Falcon	352350
Axygen MaxyClear Microtube	Axygen Scientific	MCT-175-C
Conical Screw Cap Microtube	Quality Scientific Plastics	522
Coverglass	HD Scientific	24 × 50
Fluorescent Mounting Medium	Dako	S3023
Sponge Pad for Blotting	Invitrogen	EI9052
T75 Tissue Culture Flask	IWAKI	3110-075
ZOOM® Strips pH 3-10	Invitrogen	ZM0018
10 ml Lithium Heparin Tube	BD Vacutainer	LH-367526

3. Equipment

Product	Supplier	Catalogue Number
-80°C Freezer	Sanyo	MDF-U32V
Centrifuge	Eppendorf	5430R
Chemi-Smart 5000	Vilber Lourmat	06 16314
Class II Biological Safety Cabinet	LAF Technologies	BSC 1200
Digital Microscope Camera	Leica	DFC320
Fume Hood	Conditionaire	HC-05
Heat Block	Labline	2002-LC8
Light Microscope	Olympus	BX50
Milli-Q® Biocel	Millipore	QGARD00R1
Mini BeadBeater	Biospec Products	607
Minispin Plus Microcentrifuge	Eppendorf	5424
pH Meter	Inolab	pH level 1
Power Pack	BioRad	PowerPac 300
Pressure Cooker	Russell Hobbs	RHNPC401
Raymond A Lamb Drying Hotplate	Thermo Scientific	3120061
Section Dryer	Thermo Scientific	3120064
XCell II™ Blot Module CE Mark	Invitrogen	EI9051
XCell *SureLock*™ Mini-Cell	Invitrogen	EI0001
Flow Cytometer	BD Biosciences	FACSCanto II
5% CO_2 Incubator	Flow Laboratories	220
Confocal Microscope	Zeiss	LSM 510 Meta
Microson™ Ultrasonic Cell Disruptor	Qsonica	XL 2000
Mini-PROTEAN® II Multiscreen Apparatus	Bio-Rad	170-4017
iBlot® Gel Transfer Device	Invitrogen	IB1001
ZOOM® IPGRunner™ Cassette	Invitrogen	ZM0003

4. Solutions

General Solutions

PBS (pH 7.3)

Di-sodium hydrogen phosphate	1.15 g/L
Sodium chloride	8.0 g/L
Potassium dihydrogen phosphate	0.2 g/L
Potassium chloride	0.2 g/L

TBS (pH 7.5)

Tris base	2.42 g/L
Sodium chloride	11.7 g/L

SDS Electrophoresis

Sample loading buffer

1M Tris-HCl ph 6.8	2.4 ml
20% SDS	3 ml
Glycerol (100%)	3 ml
B-mercaptoethanol	1.6 ml
Bromophenol blue	0.006 g

Store at 4^{o}C

7% Separation Gel

Water	8.5 ml
Buffer (5×)	3 ml
Acrylamide (30%)	3.5 ml
APS (10%)	150 µl
TEMED	6 µl

APS 10%: Ammonium per sulfate in ultra-pure water. Prepare always fresh.

Stacking gel

Water	8 ml
Buffer (10×)	1 ml
Acrylamide (30%)	1 ml
APS (10%)	30 µl
TEMED	12 µl

5× separation buffer (1.9 M)

Tris base	112.5 g
SDS	2.5 g
ph	8.8

Make to 500 ml

10× stacking buffer (1.3 M)

Tris base	75.5 g
SDS	5 g
ph	6.8

Make to 500 ml

<u>Western blot</u>

Running buffer Tris-glycine (pH 8.3)

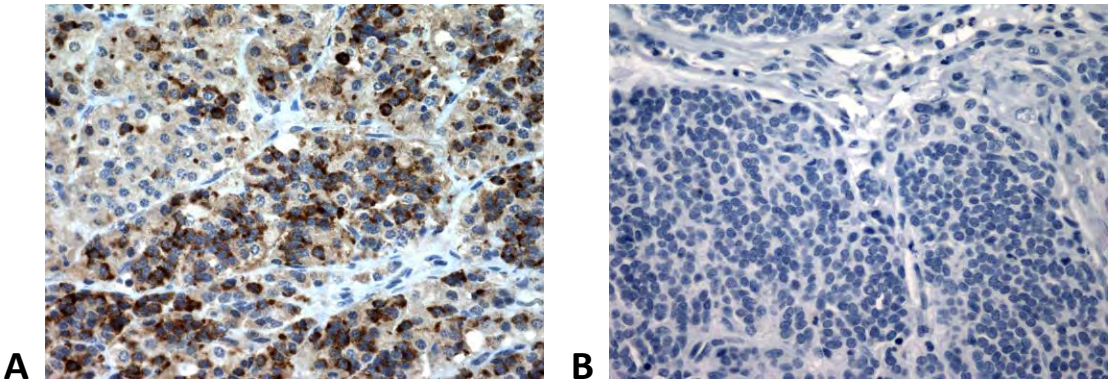
25 mM Tris base
190 mM Glycine
0.1% SDS

Transfer buffer (pH 8.3)

25 mM Tris base
190 mM Glycine
20% Methanol

Appendix 3

Chromogranin (CGA) Immunohistochemistry



Expression of CGA in normal and DFTD tissues. **A,** immunolabelling detection of chromogranin A (CGA) in cells of the devil's anterior pituitary gland. **B,** CGA immunoreactivity was not observed in devil facial tumour cells. Immunolabelling using a polyclonal rabbit anti-CGA (A0430, Dako), detection with Envision+ system (Dako) and hematoxylin counterstain.

Appendix 4

Mass Spectrometry Analysis (MudPIT)

DFTD Protein ~ 60 kDa band

1. Total proteins extracted from a DFTD cell line were separated using one dimensional SDS gel electrophoresis. A band of approximately 60 kDa was excised from the gel and proteins identified using mass spectrometry analysis (MudPIT).

Sequence Mass (Daltons)	Peptides	Unique	AA Coverage	Description
61,449	25	3	7.2%	26S proteasome non-ATPase regulatory subunit 3-like
58,746	11	3	6.7%	apoptosis inhibitor 5
66,872	12	2	4.4%	catalase-like
55,905	11	2	7.3%	coiled-coil domain-containing protein 47
67,425	3	2	4.2%	cytoskeleton-associated protein 4-like isoform 2
62,403	26	9	21.2%	dihydropyrimidinase-related protein 2-like isoform 2
62,528	31	4	10.3%	glucose-6-phosphate isomerase-like
51,882	38	5	10.6%	heterogeneous nuclear ribonucleoprotein K-like
64,532	9	2	4.9%	MAGUK p55 subfamily member 6-like
54,965	11	2	4.0%	mRNA cap guanine-N7 methyltransferase-like
55,747	64	5	14.3%	non-POU domain-containing octamer-binding protein-like
57,616	73	9	16.7%	protein disulfide-isomerase-like
70,454	25	4	8.2%	pyruvate kinase isozymes M1/M2-like
58,500	6	2	6.0%	seryl-tRNA synthetase, cytoplasmic-like
58,072	34	8	14.8%	t-complex protein 1 subunit delta
63,360	5	3	7.9%	t-complex protein 1 subunit eta-like
53,053	8	2	5.8%	t-complex protein 1 subunit zeta-like isoform 2
66,467	18	2	4.1%	WD repeat-containing protein 1 isoform 1

2. Total proteins extracted from a DFTD tumour xenografted into a NOD/SCI mouse were separated using one dimensional SDS gel electrophoresis. A band of approximately 60 kDa was excised from the gel and proteins identified using mass spectrometry analysis (MudPIT).

Sequence Mass (Daltons)	Peptides	Unique	AA Coverage	Description
55,905	7	2	7.3%	coiled-coil domain-containing protein 47
62,403	9	3	8.2%	dihydropyrimidinase-related protein 2-like isoform 2
62,528	38	5	9.2%	glucose-6-phosphate isomerase-like
51,882	24	5	13.0%	heterogeneous nuclear ribonucleoprotein K-like
64,532	12	2	4.9%	MAGUK p55 subfamily member 6-like
55,747	43	4	10.1%	non-POU domain-containing octamer-binding protein-like
56,763	4	3	5.9%	protein disulfide-isomerase A3-like
57,616	78	7	12.0%	protein disulfide-isomerase-like
70,454	22	5	10.9%	pyruvate kinase isozymes M1/M2-like
58,072	30	8	14.8%	t-complex protein 1 subunit delta
63,360	2	2	5.8%	t-complex protein 1 subunit eta-like
53,662	3	2	4.5%	vimentin-like

Appendix 5

Invitrogen ZOOM® IPGRunner System Manual

Methods Section

invitrogen™
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ZOOM® IPGRunner™ System

For isoelectric focusing of ZOOM® Strips

Catalog nos. ZM0001, ZM0002, ZM0004

Rev. Date: 00 Hs l c 2010
Manual part no. 25-0459

MAN0000256

User Manual

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